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Creating Tools to Determine Whether Katanin 60 Affects Female Rejection of Males in *Drosophila*

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A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Biology

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Abstract

During courtship, it is vital for organisms to recognize conspecifics because of the costs associated with forming interspecies hybrids. Many organisms use species-specific cues to recognize potential mates. These cues are perceived and evaluated via neural pathways. The genetic basis of how species-specific cues are evaluated and processed into receptive or rejection behaviour remains almost entirely unknown. The gene *Katanin 60* (*Kat60*) has previously been identified as contributing to interspecific mate rejection between *Drosophila melanogaster* and *D. simulans*. I use the CRISPR/Cas9 system and RNA interference (RNAi) to confirm if *Kat60* influences female receptivity between *D. melanogaster* and *D. simulans* and to identify which tissues affect it. I have created 16 RNAi stocks that silence transcripts of one species' allele, and one CRISPR stock that disrupts the *D. melanogaster* *Kat60* sequence. These stocks can be used in future reciprocal hemizyosity experiments to determine if *Kat60* affects interspecific mate rejection.

Key Words

Behavioural isolation, prezygotic, female receptivity, mating behaviour, *Drosophila melanogaster*, *Drosophila simulans*, speciation, *Katanin 60*, CRISPR/Cas9, RNA interference, Gal4/UAS.

Acknowledgements

There are many people who I want to thank for helping me get this far. I would first like to thank my supervisor, Dr. Amanda Moehring, for the endless help she has provided me over the course of this project. She always has an idea of how to proceed when a project stalls and her patience and compassion helped me stay motivated when things got difficult. I couldn't have asked for a better mentor.

I want to thank my advisory committee, Dr. Marc-André Lachance and Dr. Anthony Percival-Smith, for their valuable contributions on how to improve my project. I would also like to thank Dr. Lachance for providing feedback on my thesis. I also appreciate my examiners Dr. Kathleen Hill, Dr. Hugh Henry, and Dr. Graham Thompson for their willingness to take time out of their busy summers to help me graduate.

I wouldn't have lasted this long without the support of my labmates, who helped keep me sane during the weekends and long winter nights. In particular, I want to thank Brendan Charles, Nick Werry, Tabashir Chowdhury, Pria Mahabir, Alannah Mattice, Sebastian Heine, Akash Dhillon, and Heather Ward for being wonderful people and excellent scientists. I wish you all the best at whatever you do. My assistants Kaitlyn Dan and Pranjan Gandhi deserve special mention as I wouldn't have made half the stocks I did without their help. Thank you both for your attention to detail and great attitude; I wish I had your work ethic when I was an undergrad.

Finally, I would like to thank my family and friends for all they've done for me over the years. It really means a lot to me, and I appreciate you all suffering through my rants on CRISPR and fly sex.

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List of Abbreviations

ap: *apterous*

Cas9n: Cas9 nickase

CHC: cuticular hydrocarbon

CRISPR/Cas9: clustered, regularly interspaced palindromic repeats/CRISPR-associated protein 9

CRISPRi: CRISPR interference

dCas9: dead Cas9

DSB: double-stranded break

dsx: *doublesex*

fru: *fruitless*

gRNA: guide RNA

Kat60: *Katanin 60*

NHEJ: non-homologous end joining

mel: *Drosophila melanogaster*

miRNA: micro RNA

RNAi: RNA interference

shRNA: short hairpin RNA

sim: *Drosophila simulans*

siRNA: short interfering RNA

TBE: tris-borate EDTA

UAS: upstream activating sequence

1 Introduction

1.1 Speciation

The process of speciation, whereby distinct species arise from a common ancestor, is central to our understanding of diversity. While many different concepts are used to categorize populations as distinct species, evolutionary biologists primarily use the Biological Species Concept which states that “species are groups of actually or potentially interbreeding natural populations, which are reproductively isolated from other such groups” (Mayr 1942; reviewed in Coyne and Orr 1998). Different processes, such as selection, genetic drift, and divergent habitat utilization can contribute to speciation (reviewed in Coyne et al. 2004; Rundle and Nosil 2005). These processes can lead species to become isolated from one another by biotic factors, such as incompatible reproductive structures, or abiotic factors like mountain ranges or elevation differences (Lagomarsino et al. 2016; Barnard et al. 2017). However, reproductive isolation generally refers to genetic incompatibilities that prevent the formation of zygotes or fertile offspring between species (Gregorius 1992). Reproductive isolation can manifest as various mechanisms at all stages of reproduction. The mechanisms are commonly grouped into two categories based on when they occur in relation to the formation of the zygote: prezygotic barriers and postzygotic barriers (Chang 2004; Matute and Coyne 2010). I focus on prezygotic barriers as they are most relevant to my work.

1.1.1 Prezygotic Barriers

Prezygotic barriers refer to incompatibilities between species that prevent a zygote from being formed. Prezygotic barriers can themselves be divided into two categories: premating and postmating barriers (Markow 1997). Mechanical isolation, one example of a premating prezygotic barrier, prevents zygote formation due to physical incompatibility between reproductive organs. For example, the species pair of damselflies *Enallagma anna* and *E. carunculatum* are able to produce viable hybrid offspring but inter-specific mating rarely occurs because incompatible reproductive structures prevent males from forming tandems, which is a position required for copulation (Barnard et al. 2017). Behavioural isolation is another common form of premating prezygotic isolation. In this case, one species is not receptive to the mating

behavior of another species, as observed between *Drosophila ananassae* and *D. pallidosa*. Females of either species are less willing to mate with heterospecific males that produce a wing song during courtship relative to heterospecific males that cannot, indicating that heterospecific wing songs serve as an aversive cue for females (Yamada et al. 2002).

Postmating prezygotic barriers act during the time between copulation and zygote formation and can affect the ability of sperm from one species to reach or fertilize the egg of another (Markow 1997). While there has been relatively little research into postmating prezygotic barriers compared to premating barriers, some examples can be found across diverse taxa. Conspecific sperm precedence, where conspecific gametes outcompete heterospecific gametes, has been observed in darter species (Mendelson et al. 2007), ladybirds (Nakano 1985), and sunflowers (Rieseberg et al. 1995). Conspecific sperm precedence has also been observed between *Drosophila* species. *Drosophila simulans* can be mated to its sister species *D. mauritiana* and *D. sechellia* and produce hybrid offspring that survive to adulthood. However, *D. simulans* displays strong conspecific sperm precedence as conspecific sperm outcompetes both heterospecific sperm regardless of whether the conspecific male was mated to the *D. simulans* female before or after the heterospecific male (Price 1997). Another type of postmating prezygotic barrier includes gametic isolation, where the sperm from one species cannot survive in the reproductive tract or fertilize the egg of a heterospecific female (Turissini et al. 2017).

Behavioural isolation is often one of the first barriers to develop between sympatric species and also plays an important role in reinforcement, which is the strengthening of reproductive isolation between nascent species through selection (Coyne and Orr 1989, Noor 1995). Studies in *Drosophila* species have found that premating prezygotic barriers appear first, followed by postmating prezygotic barriers, and finally postzygotic barriers (Turissini et al. 2017). Some plant species develop prezygotic and postzygotic barriers at the same rate whereas others, such as those from the genus *Pedicularis*, developed postzygotic barriers before prezygotic ones (Moyle et al. 2004, reviewed in Widmer et al. 2009). However, most studies have found that early prezygotic isolating barrier development is most common, indicating that prezygotic isolation mechanisms tend to be the primary driver of speciation (reviewed in Coyne and Orr 2004).

1.1.2 Behavioural Isolation

Behavioural isolation is a complex phenomenon that involves multiple sensory modalities. While instances of sexual solicitations have been observed between highly diverged species (Gunst et al. 2018), species are more often unreceptive to the sexual behaviours of heterospecifics. Behavioural isolation due to inappropriate auditory information is one of the more well-known forms. The African cricket *Acanthopplus longpipes* has a slightly different calling song from a closely-related species *A. discoidales*, but *A. longpipes* females are only attracted to song models that resemble their species-specific calling song (Kowalski and Lakes-Harlan 2011). Similar results have been observed in subalpine warblers of the *Sylvia cantillans* complex, as males from both *S. (cantillans) moltoni* and *S. cantillans cantillans* were more likely to respond to songs of their own taxon (Brambilla et al. 2008). Auditory information has also been found to affect female receptivity of various *Drosophila* species: *D. melanogaster* and *D. simulans* females were more willing to mate with wingless males when a homospecific wing song was played (Ritchie et al. 1999), and *D. montana* females were willing to mate with wingless *D. lummei* males if male *D. montana* wing songs were played during the mating assay (Saarikettu et al. 2005).

Chemical cues can also act as mechanisms to recognize conspecific mates and therefore can be involved in sexual isolation between species. Female angelfish have been found to increase spawning rate in response to chemical signals produced by male conspecifics (Chien 1973), and the two swordtail species *Xiphorus nigrensis* and *X. pygmaeus* were found to prefer the odour of male conspecifics over heterospecifics (Crapon de Caprona and Ryan 1990). Pheromones have also been implicated in species-specific social interactions of rodents of the *Mus* genus (Li et al. 2013). In insects, nonvolatile pheromones consist of cuticular hydrocarbons (CHCs), waxy chemicals produced by insects that primarily serve to confer desiccation resistance (reviewed in Howard and Blomquist 2005). Because the type and amount of the CHCs differ between species, there has been considerable research into their role as behavioural isolation factors between species. For example, *Ellychnia corrusca* fireflies spent significantly more time in contact with conspecific CHC extracts relative to CHC extracts from other firefly species (South et al. 2008). Likewise, female aggression towards heterospecific males in *Gryllus campestris* crickets were

found to be influenced by their ability to identify and receive CHC chemical cues from the males (Tyler et al. 2015).

Behavioural isolation is thought to develop between two populations as a consequence of evolutionary changes within each population (Coyne and Orr 1998). Many genes that influence sexual isolation also affect other traits (such as CHCs influencing both desiccation resistance and species recognition), so selection that acts upon these genes due to one trait could cause reproductive isolation between two populations through its effect on a pleiotropic trait (reviewed in Laturney and Moehring 2012a; Nanda and Singh 2012). If the hybrids between two closely-related taxa have reduced fitness, they are selected against and reinforcement occurs. Reinforcement occurs when hybrids between incipient species have reduced fitness. This increases selection on traits in the parental species that would prevent these maladaptive matings, thus enhancing behavioural isolation (reviewed in Servedio and Noor 2003). The reduced fitness of hybrids can sometimes be due to behavioral dysfunction. For example, in two species of wolf spiders, females were far less receptive to courtship attempts from male hybrids and hybrid females had greatly reduced receptivity to all males regardless of whether they were purebred or hybrid (Stratton and Uetz 1986). Hybrid dysfunction causes reinforcement in the species pair *Drosophila persimilis* and *D. pseudoobscura*, which form sterile male hybrids as a result of impaired spermatogenesis (White 1977). Populations of these species that inhabit the same region have a much greater degree of behavioural isolation than populations that are not in contact, presumably due to the strengthening of behavioural isolation in locations where these two species come in contact and form dysfunctional hybrids (Noor 1995).

1.2 Using *Drosophila* to Study Behavioural Isolation

Drosophila species present several advantages as a model that make them ideal to study the genetic basis of behavioural isolation. *D. melanogaster* has been used in genetic research for over 100 years (Stephenson and Metcalfe 2013), and its extensive use has produced many invaluable genetic tools that can be used to identify genes of interest (Stern 2014) and create stable transgenic lines (Prokop and Root 2013).

1.2.1 The *Drosophila* Courtship Ritual

The *Drosophila* courtship ritual is a highly stereotypical series of behaviours that is common to most *Drosophila* species (reviewed in Greenspan and Ferveur 2000). The courtship ritual initiates with the male orienting himself towards the female and following her, then tapping her abdomen with his foreleg. He then “sings” her a courtship song by vibrating one wing and licks her genitalia. The male will then attempt to copulate with the female (reviewed in Sokolowski 2001). If the female deems that the male is unworthy during courtship, she will display rejection behaviours that prevent copulation (Connolly and Cook 1973). Forced copulations occur very rarely in natural *Drosophila* populations and tend to happen only in very high density environments or with extremely young adult females who cannot resist copulation attempts from mature adult males (Markow 2000). Consequently, females ultimately control whether copulation occurs in *Drosophila*.

The complexity of the *Drosophila* courtship ritual allows for the transfer of many different sensory cues between the male and female (reviewed in Laturney and Moehring 2012a). The main signals involved in courtship are visual, auditory, and chemical cues (reviewed in Greenspan and Ferveur 2000). Males primarily utilize visual stimuli to recognize females to court and these cues strongly influence how vigorously males court females. Paralyzed females elicit reduced courtship from *D. melanogaster* males and males that could not recognize horizontally-moving patterns also showed aberrant courtship behaviours (Tompkins et al. 1982). *D. melanogaster* males that lack eye pigments also have reduced mating success and have an impaired ability to orient themselves toward females, showing that visual recognition of the female is an important component of courtship in males (Connolly et al. 1969). The effect of light on courtship also varies between species. *Drosophila melanogaster* and *D. funebris* can mate easily in darkness (Hardeland 1971), *D. affinis* can copulate most easily in the light but can still mate in the dark (McRobert and Tompkins 1987), but *D. subobscura* and *D. auraria* cannot mate in the dark at all (Philip et al. 1944; Spieth and Hsu 1950).

Courtship auditory cues are most often provided through the wing vibration song the male “sings” to the female. Wing songs consist of a pulse song interrupted by a hum resembling a sine

wave (known as a sine song) of a specific frequency (Kyriacou and Hall 1982; Greenspan and Ferveur 2000). The pulse song consists of a wing beat (called a “pulse”) separated by an interpulse interval (IPI) that varies between species (Kyriacou and Hall 1982). *Drosophila melanogaster* has a pulse length of 3 milliseconds and an IPI of around 34 milliseconds whereas *D. simulans* has an IPI of 48 milliseconds (Bennet-Clark and Ewing 1968; Kyriacou and Hall 1982). Artificially producing a wing song during courtship by wingless *Drosophila melanogaster* males increases female willingness to mate (Bennet-Clark and Ewing 1968). That same study found that the species-specific nature of the song depended on IPI rather than pulse length, as *D. simulans* was significantly more likely to mate with wingless *D. melanogaster* males when the IPI of the artificial song was 48 milliseconds long.

Drosophila species are also able to recognize conspecifics by their cuticular hydrocarbon (CHC) profile. CHCs are nonvolatile chemical cues that play an important role in courtship in *Drosophila*. Because they are nonvolatile, they are only perceived at a short distance through olfactory organs or gustatory receptors on the tarsi or proboscis (reviewed in Bontonou and Wicker-Thomas 2014). CHCs are highly varied between *Drosophila* species and sometimes exhibit sexually dimorphic patterns. For example, the predominant CHC produced by *D. melanogaster* males is 7-tricosene, but *D. melanogaster* females mainly produce 7,11-heptacosadiene and produce 7-tricosene in much lower proportions (Antony and Jallon 1982). *Drosophila simulans* and *D. mauritiana* females do not produce 7,11-heptacosadiene, so this could serve as an aversive cue to males from these species (Coyne 1996). Behavioural isolation due to CHCs has been observed in both sexes of various *Drosophila* species: transferring CHCs from *D. sechellia* or *D. melanogaster* onto *D. mauritiana* females greatly reduced how often *D. mauritiana* males courted or attempted to copulate with them (Coyne and Charlesworth 1997), and transferring CHCs from *D. sechellia* or *D. melanogaster* (which both produce 7,11-heptacosadiene) onto *D. simulans* females almost completely eliminated courtship by *D. simulans* males (Coyne et al. 1994).

1.2.2 Genetic and Neurological Basis of Courtship

Because of the multiple sensory modalities and behaviours involved, the genetic and neurological bases of courtship and receptivity are highly complex. However, some genes have been discovered that influence various aspects of the courtship ritual, the aspect of this male-female interaction that has been most widely studied. The gene *desatF* encodes a desaturase in *D. melanogaster* females but not males. Knocking down this gene using RNA interference reduced the amount of diene CHCs in females and consequently led to a decrease in courtship and copulation attempts by *D. melanogaster* males (Chertemps et al. 2006). Kyriacou and Hall (1982) found that the *period* gene, which encodes a transcription factor known to play a role in circadian rhythms, influenced the regularity of courtship songs in *D. melanogaster*. *Drosophila melanogaster* and *D. simulans* females were also more likely to copulate with wingless *D. melanogaster* males when an artificial wing song was played that matched the species of the female (Kyriacou and Hall 1982), though these results are controversial due to difficulty replicating them (Stern 2014; Kyriacou et al. 2017; Stern et al. 2017a).

Two particularly important genes involved in *Drosophila* courtship are *fruitless* (*fru*) and *doublesex* (*dsx*). Both *fru* and *dsx* encode transcription factors and are able to change the structure of the fly central nervous system (CNS) dramatically in a sex-specific manner due to their sex-specific mRNA splice products (Ryner et al. 1996; reviewed in Dauwalder 2011). The male forms (Fru^M and Dsx^M) of these transcription factors produce the neuronal circuitry that encode male specific behaviours whereas female-specific neural circuitry is controlled only by the female Dsx^F (Rideout et al. 2010; reviewed in Dauwalder 2011; Pavlou and Goodwin 2013). Approximately 1500 neurons in the CNS express the male-specific form of *fruitless*, and many are in regions implicated in sexual behaviour (Lee et al. 2000). Fru^M is also expressed in sensory neurons outside of the CNS, for example olfactory receptor neurons and gustatory receptor neurons, though it is not expressed in the sensory neurons of the visual system (Stockinger et al. 2005). Stockinger et al. (2005) also proposed that these Fru^M -expressing neurons are organized into a circuit which is thought to affect male courtship behaviours.

Fru^M neurons have been strongly linked to behaviour: silencing expression of Fru^M transcripts has been shown to impair male courtship and expressing Fru^M in females causes them to exhibit male courtship behaviours (Ito et al. 1996; Demir and Dickson 2005; Stockinger et al. 2005). The 1500 *fru*-expressing neurons can be differentiated into *ca.* 100 anatomically-distinct types in the *Drosophila* CNS, with a particularly high concentration of *fru*-expressing neurons surrounding the mushroom body (Yu et al. 2010). The mushroom body of the *Drosophila* brain has been linked to memory and sensory information integration (reviewed in Guven-Ozkan and Davis 2014). A recent study found that each lobe of the mushroom body integrates a different combination of sensory modalities (Yagi et al. 2016), and certain lobes affect courtship behaviours. The gamma lobes of the mushroom body contain many *fru*-expressing neurons (Yu et al. 2010) and silencing the neurons in these lobes impairs courtship memory (Montague and Baker 2016).

While *fru* mutations in males can nearly completely prevent courtship from occurring, *dsx* mutations do not affect courtship as strongly but still have a considerable effect. Males that lack a functional copy of *dsx* are unable to sing sine song and show other defects in song production. *dsx* is expressed in a specific subset of neurons known as TN1A neurons, which are required for males to produce the sine song (Villegla and Hall 1996; Shirangi et al. 2016). TN1A neurons share a strong functional connection with the *hgl* motoneuron, which is also required for the production of sine song (Shirangi et al. 2013). Shirangi et al. (2016) found that TN1A neurons require expression of *dsx* during development to make proper synaptic contacts with the *hgl* motoneuron, proving that *dsx* plays an important functional role in male wing song production.

Female receptivity, while less studied, is known to be influenced by some of the same mechanisms that affect male courtship. Silencing *fru* neurons in females causes them to reject male copulation attempts significantly more often and also to lay more eggs, which are both stereotypical postmating behaviours (Kvitsiani and Dickson 2006). Genes *fru* and *dsx* both mediate the decrease in postmating female receptivity caused by the transfer of male sex peptide in male ejaculate. Expressing the membrane-bound form of sex peptide in *dsx*-expressing neurons caused virgin females to exhibit reduced receptivity to males to levels comparable with mated females. The cell bodies of these neurons were found in the abdominal ganglion and

projected both to the suboesophageal ganglion in the brain and to the uterus, providing a neural link between sex peptide and female postmating behaviours (Rezával et al. 2012). The gene *dsx* has also been found to have a direct effect on female receptivity: expression of *dsx* neurons in the pC1 and pCd neuronal clusters significantly increased female receptivity whereas silencing those clusters reduced receptivity. The pC1 neuronal cluster was found to respond to artificial pulse and sine songs and the male pheromone cVA whereas pCd only responded to cVA (Zhou et al. 2014). Some genes affect female receptivity through neural regions associated with *fru* expression. For example, female *spinster* mutants are known to reject copulation attempts by males vigorously (Suzuki et al. 1997). These rejectionary behaviours were found to be mediated by the VA1v glomerulus of the antennal lobe and the spin-A cluster in the suboesophageal ganglion (Sakurai et al. 2013). The VA1v glomerulus is one of the sexually-dimorphic brain regions that *fru* neurons project to (Stockinger et al. 2005) and the suboesophageal ganglion has been shown to affect female receptivity through *dsx*-expressing neurons (Rezával et al. 2012).

dsx and/or *fru*-expressing neurons are not the only ones that influence mating behaviour, however. For example, the *apterous* (*ap*) gene encodes a transcription factor that influences wing and neuron pathway development (Lundgren et al. 1995), and female *ap*-deficient mutants were found to exhibit reduced female receptivity (Ringo et al. 1991). However, the *ap* neurons responsible for this reduction in mating were not part of a sexually differentiated circuit and did not express either *fru* or *dsx* (Aranha et al. 2017).

Despite great progress in our understanding of the genetic and neural basis of female receptivity in *Drosophila*, much remains unknown. One particularly important question for evolutionary biologists working with *Drosophila* is whether the genes and neural regions that affect female receptivity towards males of the same species affect heterospecific males as well. Unfortunately, as of the time of writing, no genes have been identified that are involved with interspecific mate rejection in females (reviewed in Laturney and Moehring 2012a). As such, we have a limited understanding of how behavioural isolation develops between species on a genetic level.

As mentioned earlier, many aspects of the *Drosophila* courtship ritual have species-specific components, and females ultimately decide whether a mating attempt is successful. Therefore, when searching for genes that affect interspecific mating, it makes sense to identify them through

changes in female receptivity towards mating attempts from heterospecific males. Identifying genes that influence female receptivity to courtship by other species would greatly advance our understanding of how behavioural isolation develops and speciation in general.

1.3 Genetic Basis of Behavioural Isolation in *Drosophila*

Though there has been extensive research into behavioural isolation, the genetic basis is still unclear. Reproductive isolation in *Drosophila* is a complex polygenic trait involving a number of concurrently evolving loci (Zeng et al. 2000; Ting et al. 2001; McNabney 2012). For example, a QTL mapping study investigating the genetic basis of male genital arch shape differences between *D. simulans* and *D. mauritiana* found 19 different loci that contributed to the trait (Zeng et al. 2000). Incompatible reproductive structures could act as unattractive tactile cues that reduce female willingness to mate (Barnard et al. 2017). In addition, Ting et al. (2001) found that seven loci on the third chromosome influenced either female preference or male mating success in different races of *D. melanogaster*. The authors believe that the influence of each region on genital arch shape is epistatic or polygenic, further complicating the ability for scientists to determine specific genes that influence behavioural isolation. Some progress has been made towards identifying genes of interest that influence reproductive isolation, however: the *desaturase2* gene affects desiccation resistance in two races of *D. melanogaster* and may have influenced behavioural isolation between them through ecological adaptation (Greenberg et al. 2003). No individual genes responsible for behavioural isolation have been identified at the time of writing, but QTL and deficiency mapping studies have identified the third chromosome as a promising region of study (Civetta and Cantor 2003; Moehring et al. 2006; Laturney and Moehring 2012b).

Genes that cause behavioural isolation are difficult to identify for several reasons. One of the biggest obstacles to overcome is that hybrids of different species are difficult (and sometimes impossible) to produce, and surviving offspring are often sterile. Sterile hybrids can prevent researchers from introgressing genes into either parental genome and can make QTL mapping impossible (reviewed in Laturney and Moehring 2012a). This problem disproportionately affects genes that cause premating isolation because it is still possible to study sterility or inviability

genes in parental species that cannot produce fertile hybrids (Coyne and Charlesworth 1989; Sawamura et al. 1993; Ting 1998; Chatterjee et al. 2007). Despite these difficulties, the powerful genetic tools available to drosophilists and the well-characterized mating behaviour of *Drosophila* species makes them an excellent system with which to study behavioural isolation.

1.3.1 Behavioural Isolation between *D. melanogaster* and *D. simulans*

D. melanogaster and *D. simulans* (henceforth referred to as *mel* and *sim*) are two commonly-used species in the study of species isolation. The species diverged about 5.4 million years ago and currently exist sympatrically (Tamura et al. 2004). *D. melanogaster* might initially appear to be a poor model to study behavioural isolation genes as it only forms sterile or inviable hybrids with other species (reviewed in Orr 2005). However, the effect of single genes on behavioural isolation can be studied in sterile F1 hybrids by crossing single gene knockout lines to a different species and studying the effect on courtship and copulation in the hemizygous hybrid (Stern 2014). *D. simulans* females are extremely choosy towards *mel* males whereas the reciprocal heterospecific cross is far easier to produce (Sturtevant 1920). Offspring produced from the permissive cross are always female and always sterile. Two genes, *Lethal hybrid rescue* (*Lhr*) and *Hybrid male rescue* (*Hmr*) interact to cause this hybrid male lethality (Brideau et al. 2006). The two genes code for proteins that repress expression of transposable elements and regulate satellite DNA transcription, suggesting that lethality might be caused by misregulation of these elements (Satyaki et al. 2014). Despite being nearly indistinguishable visually, *sim* and *mel* exhibit differences that could serve as aversive cues for females, such as the different CHC blends in the two species (Antony and Jallon 1982; Sharma et al. 2012), and the different IPI of the wing song (Bennet-Clark and Ewing 1968a), mentioned above.

Even though *sim* females strongly reject *mel* males, hybrid females are somewhat receptive to courtship attempts by *mel* males (Davis et al. 1996). This suggests that the *mel* alleles for receptivity towards *mel* males are semi-dominant over the *sim* alleles for rejecting these males. Because *mel* males readily court *sim*, *mel*, and hybrid females, genes responsible for female receptivity towards heterospecific males can be identified through knocking out the *mel* allele of

a gene in a hybrid female and quantifying the resulting effect on receptivity to *mel* males. If the gene is responsible for receptivity, then the *sim* rejection phenotype should be at least partially restored. The genes influencing female rejection of heterospecific males could normally be identified through recombination mapping, but recombination mapping requires fertile F1 hybrids that *mel* is unable to produce (reviewed in Orr 2005). This problem can be overcome by using deficiency mapping and balancer chromosomes. Deficiency mapping and balancer chromosomes allow researchers to test if certain genomic regions influence a quantitative trait (such as female rejection) while only requiring F1 offspring. These techniques therefore allow scientists to utilize the many genetic tools available in *D. melanogaster* to study behavioural isolation.

1.4 Genetic Tools Available in *D. melanogaster*

Because *D. melanogaster* is one of the most widely-used model organisms, many different researchers have developed tools to help identify or characterize genes. *D. melanogaster* was one of the first organisms to have its genome fully sequenced (Adams et al. 2000) and many well-studied phenotypic markers can be used to track a gene of interest (reviewed in Gramates et al. 2017). Some tools, such as the Gal4/UAS system or green fluorescent protein, originated in other organisms but have been adapted for use in *D. melanogaster* (Brand and Perrimon 1993; Plautz et al. 1996). New transgenic lines can also be created easily and quickly due to the efficiency of *Drosophila* blastoderm injections. Deficiency mapping can be done especially well in *D. melanogaster* as available deletion stocks cover 98.3% of all euchromatic genes (Bloomington *Drosophila* Stock Centre). Many deleted regions are homozygous lethal and would normally be lost from a stock due to selection. However, homozygous lethal deletions can be maintained over balancer chromosomes which allow *Drosophila* geneticists to maintain mutations over generations without fear that they will be lost due to selection or recombination.

1.4.1 Balancer Chromosomes

Balancer chromosomes (referred to as balancers) are composed of three key components: multiple inversions, homozygous-lethal mutations, and dominant visible phenotypic markers

(reviewed in Kaufman 2017). Multiple inversions in balancers prevent crossing over from occurring near inversion breakpoints and cause lethality in all offspring arising from gametes that underwent single crossover events within inversions due to massive aneuploidy (Bloomington *Drosophila* Stock Centre). Balancers also include recessive lethal mutations and dominant visible markers which allow researchers to track the presence or absence of a balancer through generations and allowing stocks to maintain a mutation of interest (Prokop and Root 2013). Balancers are most effective when the mutation of interest is very close to an inversion breakpoint to reduce the possibility of losing it through double crossover events (reviewed in Kaufman 2017). Because harmful mutations cannot easily be lost through selection when balanced, balancer chromosomes can allow for the maintenance of highly deleterious alleles or deletions, such as those that cause inviability or sterility when homozygous. Balancers also allow for the creation and maintenance of deletion stocks that can be used for deficiency mapping.

1.4.2 Deficiency Mapping and Reciprocal Hemizyosity

Deficiency mapping is a technique used to identify small genomic regions that influence some recessive quantitative trait of interest. Deficiency mapping utilizes *D. melanogaster* stocks that have known regions of their genome deleted and maintained over a balancer. This means that the stock is effectively hemizygous at known loci. When mapping for a recessive trait, such as eye colour, deletion stocks can be crossed with other lines that contain the mutant phenotype to identify where the gene(s) of interest are located. If genes influencing the trait are not located within the deletion, they will be masked by the wild-type allele present on the deficiency chromosome and the organism will resemble the wild-type. If the genes influencing the trait are located within the deletion, however, there will be no wild-type allele to mask the effect of the mutation and the fly will display the mutant phenotype in a manner similar to classic complementation tests (Figure 1A). Multiple deficiency lines can be used to narrow the region of interest. Deficiency mapping has been used in *Drosophila* to identify regions associated with longevity (Pasyukova et al. 2000), female receptivity (Laturney and Moehring 2012b), and canalization of developmental processes (Takahashi et al. 2011), among other traits. Once candidate regions have been identified, the genes within can be tested by crossing the strain of interest to stocks containing loss-of-function mutations for those genes then testing in a similar

manner as deficiency mapping. If the same mutant phenotype is observed after that cross, then that candidate gene at least partially influences the trait of interest.

The reciprocal hemizyosity test is a variant of deficiency mapping that aims to determine the relative contribution of strain- or species-specific alleles on a trait (Stern 2014). Briefly, a deletion is generated in both strains to be tested, and then the deficiency line from one strain is crossed with the wild-type of the other. This produces a line that is hemizygous at a specific locus, which unmasks the alleles of one line at that locus. The effect of alleles within that region on a trait can then be determined in an identical manner to deficiency mapping. The reciprocal cross is then performed and the effect on the trait is once again characterized. Any difference between the reciprocal deletion lines represents the contribution of alternative alleles due to differences between strains, as the genetic background is otherwise identical (Figure 1B). This strategy can be applied to two different species so long as they are able to produce interspecies F1 hybrids. Reciprocal hemizyosity tests are therefore very well suited to characterizing genes that influence behavioural isolation between *mel* and *sim*. Because *sim* alleles for rejection of *mel* males are recessive towards the *mel* alleles, identifying regions that restore *sim*-like rejection behaviours when hemizygous in hybrids could help find genes that cause behavioural isolation between *mel* and *sim*. The reciprocal test acts as a control, as removal of the recessive *sim* allele should have no effect on receptivity in hybrids. Previous work in my lab used deficiency mapping to identify five small regions on the right arm of the third chromosome that influenced this behaviour, and further fine-scale deficiency mapping identified *Katanin 60* as a candidate gene for behavioural isolation between *mel* and *sim* (Laturney and Moehring 2012b; Calhoun 2017).

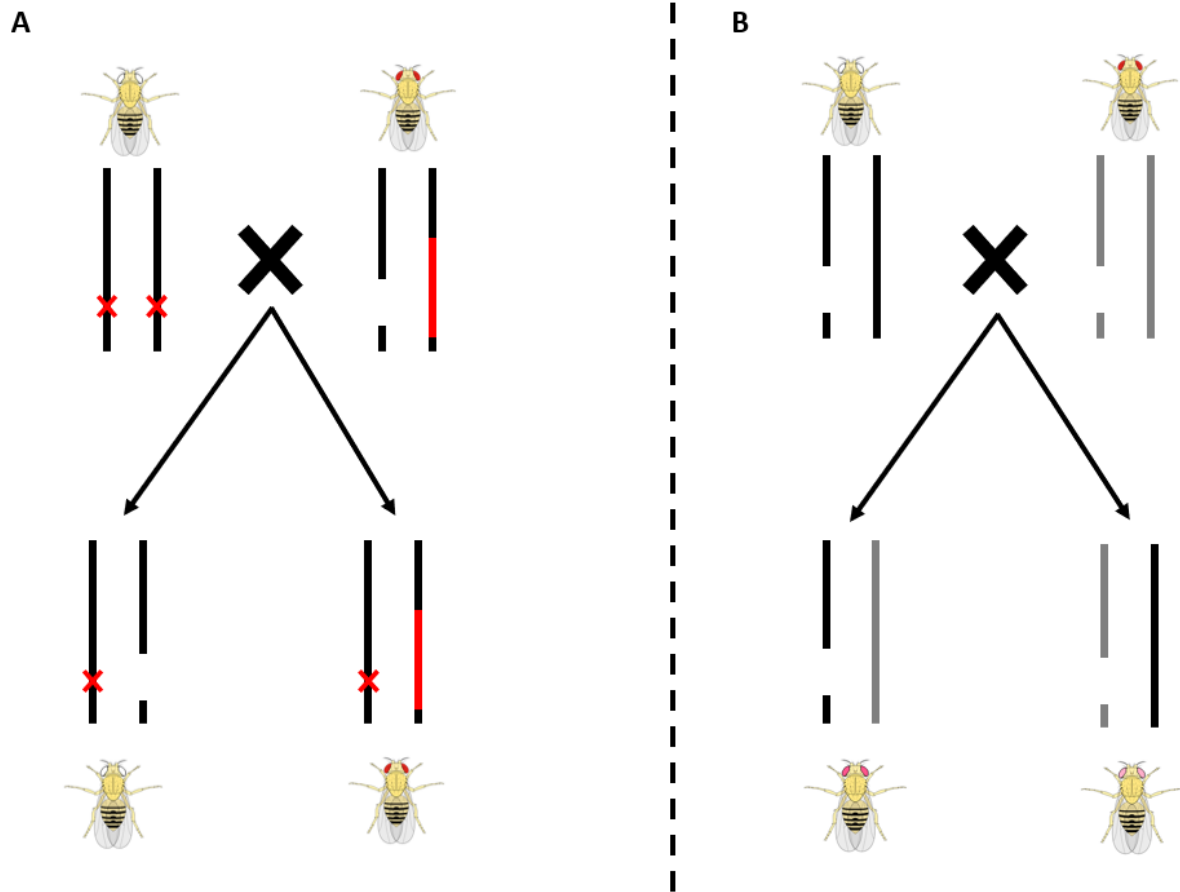


Figure 1: Illustration of deficiency mapping and the reciprocal hemizyosity test.

A) Deficiency mapping is used to identify the location of a gene that influences a recessive trait (white eyes, in this case). A strain that displays the mutant phenotype is crossed to a deficiency stock that contains a deletion maintained over a balancer (inversions on the balancer are indicated by the red region). If the region that has been deleted contains a gene that influences the phenotype, then offspring containing the deletion will display the mutant phenotype as the mutant allele will be unmasked. **B)** The reciprocal hemizyosity test is used to determine the relative contribution of strain- or species-specific alleles to a trait (eye colour is used in this example). Deletions are created in both strains/species and these deletion lines are crossed together. This produces deletion lines that are hemizygous only at the deleted region. This unmasks the alleles of one lines and the contribution of genes in that region can be quantified and compared to the reciprocal deletion line to determine the relative contribution of line-specific alleles to a phenotype.

1.5 Determining if *Katanin 60* Affects Female Receptivity

Katanin 60 (henceforth *Kat60*) encodes an ATPase associated with diverse cellular activities. More specifically, *Kat60* encodes the catalytic (called AAA+) domain of the microtubule-severing protein katanin (Hartman et al. 1998). The katanin protein is a heterodimer composed of two subunits, named p60 and p80 due to their respective size in kilodaltons (Yu et al. 2005). The p60 subunit, encoded by *Kat60*, contains the catalytic domain that severs microtubules, whereas the p80 subunit is responsible for localizing the enzyme to the centrosome and regulating its activity (Hartman et al. 1998; Grode and Rogers 2015). Katanin preferentially severs microtubules at the plus end, destabilizing them and inducing microtubule ‘catastrophes’ (transitions from growth to shrinkage) (Díaz-Valencia et al. 2011).

Kat60 is expressed during all embryonic and larval stages and in certain adult tissues, such as the gut, ovary, and brain (Tomancak et al. 2002, 2007; Chintapalli et al. 2007; Hammonds et al. 2013). *Drosophila melanogaster* is unable to survive to adulthood without a functional copy of *Kat60* (Mao et al. 2014). This is likely because Katanin is required for correct cellular function, as cells lacking *Kat60* divide abnormally due to *Kat60*’s role in chromosome motility during anaphase of mitosis. Chromatid migration during anaphase in cells that are silencing expression of *Kat60* is so slow that chromosomes begin decondensing before segregation is complete (Zhang et al. 2007). *Kat60* has also been found to double migration rate of *Drosophila* cells when silenced (Zhang et al. 2011). While all cell types require katanin, it is especially important in neurons. NDEL1, a protein implicated in human lissencephaly, interacts with and regulates the distribution of the p60 subunit (Toyo-Oka et al. 2005) and katanin also regulates microtubule release and length in neurons (Ahmad et al. 2000). *Drosophila melanogaster* neurons lacking a functional copy of *Kat60* have neurotransmission defects, increased synaptic boutons, and increased dendrite length and branching (Mao et al. 2014).

Katanin has previously been implicated in speciation processes. Katanin is twenty times more active in *Xenopus tropicalis* than *X. laevis* which results in longer kinetochore fibers in *X. laevis* eggs. This difference in activity causes sperm and egg incompatibilities that prevent the formation of viable interspecies zygotes (Loughlin et al. 2011). A *Kat60* ortholog was also found to be noticeably different between two closely-related species of killifish and was thought to be involved in possible spermatogenesis differences between them (Kozak et al. 2014), which could potentially give rise to sterility in interspecies hybrids. Katanin affects spermatogenesis in mice and humans: the p80 subunit of katanin was localized to type B spermatogonia and stage IV and V spermatocytes in humans (Pleuger et al. 2016).

Kat60 is particularly interesting because it has the potential to influence both prezygotic isolation (through behavioural isolation) and postzygotic isolation (through hybrid inviability). Because the amount of *Kat60* can greatly affect neuron development (Mao et al. 2014), it is possible that different expression patterns of *Kat60* between *mel* and *sim* influences how some sensory cues obtained during courtship are interpreted. Previous work in my lab found that *Kat60*'s effect of female receptivity was mediated by auditory cues (Calhoun 2017). *Kat60* could therefore affect female receptivity by altering the structure of brain regions such as the Johnston organ or the antennal mechanosensory and motor center, which are involved in auditory information processing (Kamikouchi et al. 2009). *Kat60*'s effect on behavioural isolation between *mel* and *sim* can be determined by creating *Kat60* deficiency lines in both species and using the reciprocal hemizygoty test to see if an allele of *Kat60* affects the mating behaviour of female hybrids. We also need to determine where *Kat60* is acting in the fly to cause behavioural isolation between *mel* and *sim*. This can be done by knocking down *Kat60* mRNA transcripts in particular tissues and testing if this affects mating behaviour. My project's goal was to create *Kat60* deficiency lines using the CRISPR/Cas9 system and to create targeted *Kat60* transcript knockdown lines by utilizing RNA interference (RNAi) in conjunction with the Gal4/UAS system.

1.5.1 Creating *Katanin 60* Deficiency Lines Using CRISPR/Cas9

Many tools can be used to produce single-gene deficiency lines, but the CRISPR/Cas9 (clustered, regularly interspaced palindromic repeats/CRISPR-associated protein 9) system is one

of the most versatile and efficient gene editing tools available to geneticists. The CRISPR/Cas9 system was derived from the adaptive immune system of the bacterium *Streptococcus pyogenes* (reviewed in Mei et al. 2016). There are two classes of CRISPR-Cas systems comprising six types (known as types I through VI), and each type is further divided into subgroups (reviewed in Makarova et al. 2015; Zhang and Ye 2017). Class 1 CRISPR-Cas systems use a multisubunit protein complex to cleave targets whereas class 2 CRISPR-Cas systems only require a single protein (reviewed in Makarova et al. 2015). Types IV, V, and VI are a relatively recent discovery and are poorly characterized as of the time of writing. Types I and II cleave DNA while type III is thought to cleave both DNA and RNA (Brouns 2008; Tamulaitis et al. 2014; reviewed in Hille and Charpentier 2016). Because type II CRISPR-Cas systems only require a single protein (Cas9) to cut DNA, they have become the preferred system to use in the lab.

The Cas9 protein contains several structural features that allow it to cleave target DNA precisely (Figure 2). The proto-spacer adjacent motif (PAM)-interacting domain screens for NGG nucleotide sequences, which are required for Cas9-DNA binding (Mojica et al. 2009). Cas9 has nucleotide binding domains that allow it to interface with the guide RNA (gRNA)-target DNA complex and two endonuclease domains, RuvC and HNH (reviewed in Oakes et al. 2014). The Cas9 protein works by forming a complex with a guide RNA which hybridizes with a perfectly complementary DNA target. The Cas9 protein will then bind to the PAM and cleave the target DNA three bases upstream of it, inducing a double-stranded break (DSB) (reviewed in Oakes et al. 2014; Wu et al. 2014). In bacteria, the DSBs produced by Cas9 are an effective defence against invasive nucleotides from bacteriophages. In eukaryotic cells, however, these double stranded breaks are repaired using non-homologous DNA end joining (NHEJ). NHEJ is less accurate than homology-directed repair (HDR) of DNA breaks but is often necessary in situations where homologous DNA is not readily available (reviewed in Lieber et al. 2003). NHEJ repair can result in the loss of up to 14 base pairs or even small insertions at either end of the break (reviewed in Lieber 2010), which can potentially knock out a gene by causing indels and frame shifts. Larger deletions can be induced by using two gRNA molecules that target areas close to one another, as Cas9 will induce DSBs at both sites and excise the intervening region (Canver et al. 2014). Injecting plasmid DNA or a single-stranded DNA oligo that contains sequence homology to both ends of the DSB along with the gRNA and Cas9 can induce HDR

rather than NHEJ. The incidence of HDR can also be enhanced by suppressing enzymes involved in NHEJ (Chu et al. 2015). This technique can be used to insert DNA of interest at a precise point on the genome or even replace native DNA entirely (Richardson et al. 2016; Zhang et al. 2017).

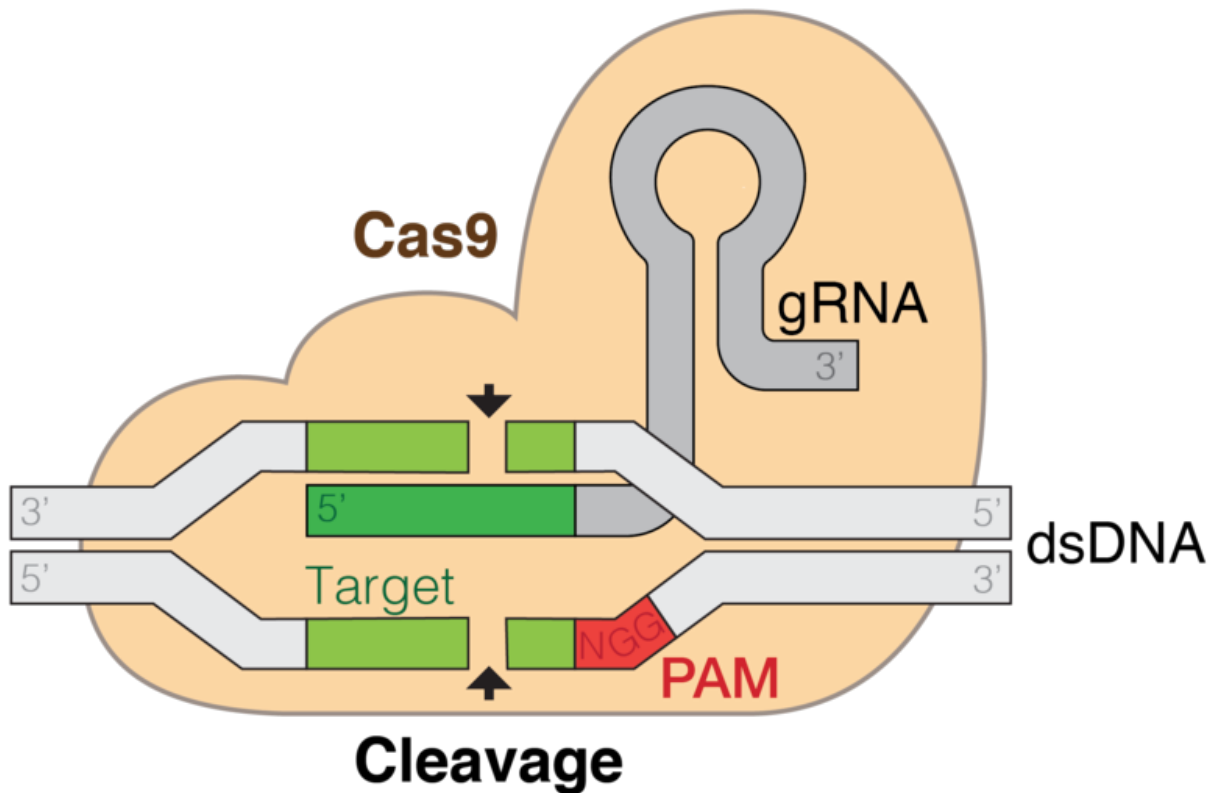


Figure 2: Cas9 inducing a double-stranded break in a target sequence.

The Cas9 protein forms a complex with a guide RNA molecule (gRNA) that contains a perfectly complementary sequence (dark green) to a DNA target (light green). Cas9 can identify the target site through hybridization of the gRNA to the DNA sequence and through a protospacer-adjacent motif (PAM) contained within the target site. Upon recognizing the target and the PAM, Cas9 induces a double-stranded break in the DNA. Image courtesy of Marius Walter (Wikimedia Commons free media repository).

Scientists have modified the function of Cas9 to improve specificity or to change how it interacts with the target. The gRNA was modified into a single synthetic oligo instead of the two RNA molecules that the bacteria normally use (Jinek et al. 2012), and further modifications to the size of the gRNA can influence specificity (Fu et al. 2014). The RuvC and HNH endonuclease

domains of the Cas9 can be deactivated with point mutations to produce Cas9 proteins that can only cut one strand (Cas9 nickase or Cas9n) or are catalytically dead (dCas9). Although Cas9n only causes single-stranded breaks at the target site, deletions can still be generated by injecting two gRNA molecules that target nearby regions on opposite strands. This method is less efficient than using normal Cas9, but this strategy greatly reduces off-target effects as two cuts are needed to induce DSBs and single nicks are easily repaired by cellular machinery (Ran et al. 2013; Wu et al. 2014). dCas9 proteins bind to targets but are unable to cleave them. This can result in transcriptional interference and gene silencing as RNA polymerase is physically blocked by the dCas9 protein (Qi et al. 2013). dCas9 proteins can be further modified to reversibly control gene expression: dCas9 proteins fused to transcriptional activators or repressors can activate or inhibit gene expression, respectively (reviewed in Kampmann 2018).

I utilized the CRISPR/Cas9 system to knock out one copy of *Kat60* in *mel* and *sim* to produce hemizygous individuals. These hemizygous individuals can then be mated with the other species to produce hybrids with only a functional *mel Kat60* allele (*Kat60^{mel}*) and hybrids with only a functional *sim Kat60* allele (*Kat60^{sim}*). The remainder of the genome will be identical in these two types of hybrids, controlling for background genetic effects. The effect of genes on heterospecific courtship can be determined by using a reciprocal hemizyosity test (Figure 1B; Stern, 2014). If hemizygous hybrid females containing only *Kat60^{mel}* are more willing to mate with *mel* males than hybrid females bearing only *Kat60^{sim}*, then *Kat60* affects heterospecific rejection.

1.5.2 Finding Where *Kat60* Acts using RNAi and the Gal4/UAS System

The CRISPR/Cas9 system is well suited to inducing mutations into a gene early in the development of an organism to create a random genetic mosaic of mutated and unmutated cells. It is also commonly used to induce mutations in germline cells to ensure that every cell of the resulting offspring contains the desired mutation. While this is useful to study the broad-scale effects of a mutation, it cannot be used to study the effect of a gene at specific developmental stages or in specific tissues. This can often be done by knocking down the transcript of the gene rather than knocking out the gene itself. RNA interference (RNAi) is one of the most effective

techniques for reversibly knocking down mRNA transcripts when working with *D. melanogaster*.

RNAi was first reported in 1986 by scientists attempting to breed petunias (Ecker and Davis 1986), but the double-stranded RNA molecules used in current gene silencing experiments were discovered in 1998 by Fire et al., who observed that double-stranded RNA interfered with protein expression far more than single-stranded RNA molecules in *Caenorhabditis elegans*. There are three different types of RNAi (miRNA, siRNA, and shRNA) but all are eventually processed into 21 or 22 nucleotide-long fragments of double-stranded RNA (Elbashir et al. 2001). Micro-RNA (miRNA) are transcribed into primary miRNA molecules which are processed into pre-miRNAs by the proteins Drosha and Pasha (Figure 3A) (reviewed in Torrecilla et al. 2014; Tran and Montano 2016). Pre-miRNAs are then exported into the cytoplasm by exportin 5 where Dicer further processes the pre-miRNA into mature miRNA (Lund and Dahlberg 2006). The antisense strand, which can be differentiated from the inert passenger strand by a 2 nucleotide overhang, guides the RNA-induced silencing complex (RISC) to complementary mRNA which it then cleaves (reviewed in Hammond 2005; Torrecilla et al. 2014). Short interfering RNA (siRNA) molecules are synthetic dsRNA constructs that are injected into the cytoplasm of the cell. As they do not need to be exported out of the nucleus, only Dicer processes them before they are incorporated into RISC (Figure 3B). Short hairpin RNAs (shRNA) inhibit mRNA translation in a similar manner to siRNA, but require nuclear processing like miRNAs (Figure 3C) (reviewed in Torrecilla et al. 2014). shRNAs are introduced into the nucleus where they are transcribed and then processed by Drosha into pre-shRNAs (Rao et al. 2009). They are then transported into the cytoplasm by exportin 5 and processed into siRNA by Dicer. After processing by Dicer, they are incorporated into RISC and bind to the target mRNA. Micro-RNA are usually produced by endogenous genes and bind to a wide range of targets as they only require ~7 complementary nucleotides to bind effectively (Brennecke et al. 2005; reviewed in Dai et al. 2012). Short interfering RNAs and shRNAs usually only silence mRNA transcripts containing regions of perfect complementarity but they have been known to produce off-target effects, particularly when the 7 nucleotides of the 5' "seed region" are complementary to other transcripts (Lin et al. 2005; Cullen 2006; Jackson 2006).

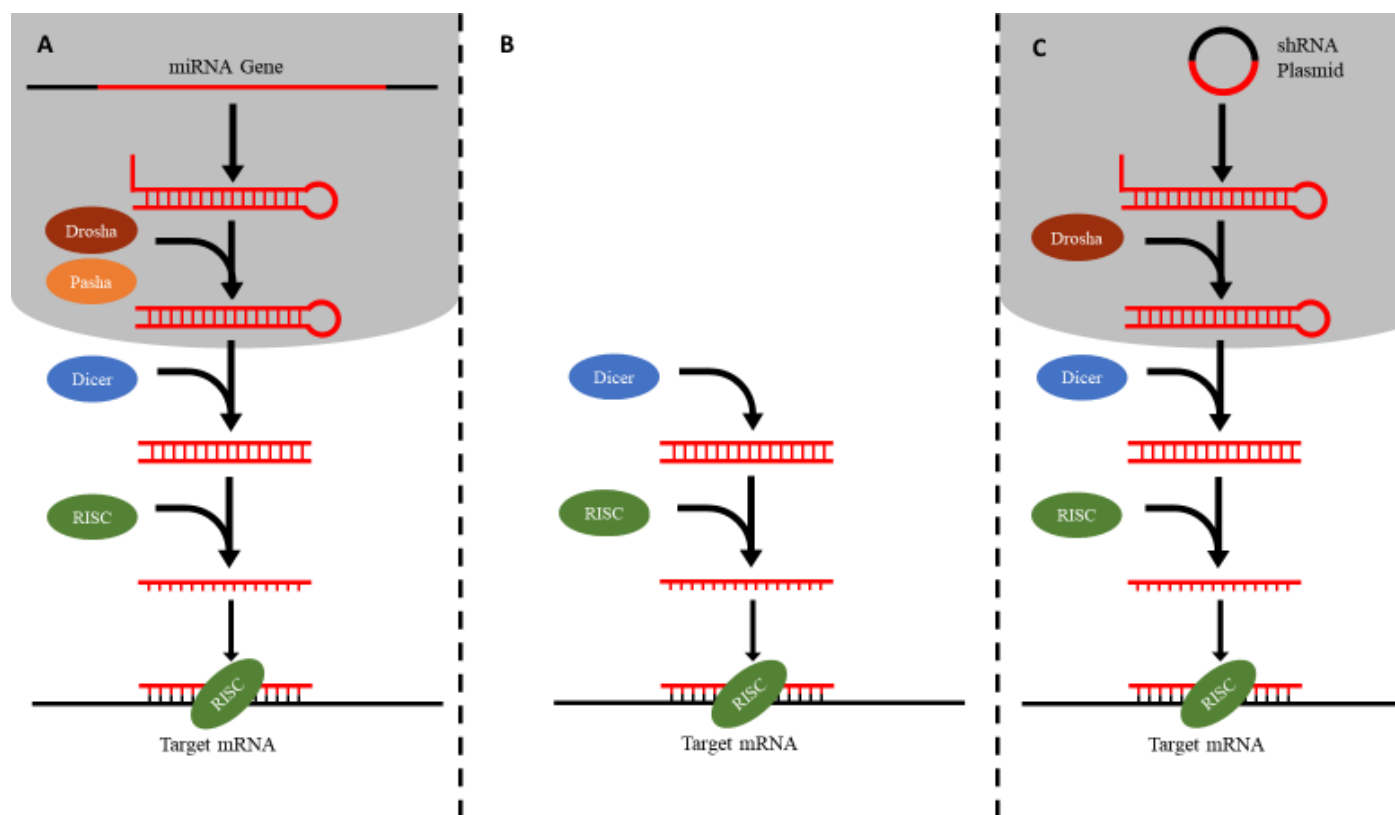


Figure 3: Processing of miRNA, siRNA, and shRNA.

An illustration of how the three different types of double-stranded RNA molecules are processed. Shaded regions indicate steps that take place in the cell nucleus. **A)** Micro-RNA (miRNA) genes are transcribed in the nucleus into primary miRNA. These molecules are processed by Drosha and Pasha into pre-miRNA and exported out of the nucleus. Dicer unwinds the pre-miRNA and separated the active antisense strand from the passenger strand. The antisense RNA is then incorporated into the RNA-induced silencing complex (RISC) which then finds and cleaves complementary mRNA. **B)** Short interfering RNA (siRNA) is injected into the cytoplasm of the cell and the antisense strand is separated from the passenger strand by Dicer. The antisense strand is then loaded into RISC and used to find and cleave complementary mRNA molecules. **C)** Short hairpin RNA (shRNA) genes on plasmids are transcribed in the nucleus into primary shRNA. Drosha converts this into pre-shRNA and the pre-shRNA is unwound and processed by Dicer after it is exported out of the nucleus. The antisense RNA strand is then incorporated into RISC and used to silence complementary mRNA.

RNAi provides several benefits over gene editing techniques such as the CRISPR/Cas9 system. As previously mentioned, CRISPR/Cas9 is unable to cause transient knockout of a gene. This can be problematic if the gene is required for cell viability or if researchers want to study the effect of a gene at a specific developmental stage (reviewed in Unniyampurath et al. 2016). A

newer version of CRISPR, called CRISPR interference (CRISPRi), where the gRNA can be reversibly bound to a target gene without splicing, allows targeted knockdown of transcription. While CRISPRi causes fewer off-target effects than RNAi (Gilbert et al. 2014; Smith et al. 2017), the silencing machinery is not produced endogenously and therefore might only be effective for a short duration after injection. RNAi is a more widely-used technique relative to CRISPRi, so it is far easier to obtain or produce RNAi lines. Despite this, the CRISPR/Cas9 system also presents some advantages over RNAi. The CRISPR/Cas9 system can knock out gene function in all cells of an organism which completely eliminates expression. RNAi is unable to provide this robust level of gene silencing, and efficiency of transcript knockdown can vary a great degree (reviewed in Boutros and Ahringer 2008). RNAi also must target the mature mRNA transcript whereas the CRISPR/Cas9 system can target regulatory sequences to study their function. The *Drosophila* RNAi Screening Center (DRSC) and Transgenic RNAi Project (TRiP) are particularly useful resources, as they have produced many RNAi lines and vectors that can be easily purchased for use. One such vector is pVALIUM20 (Ni et al. 2011), which pairs RNAi with the Gal4/UAS system for tissue-specific gene silencing.

The Gal4/UAS system has been used in *D. melanogaster* to induce high levels of gene expression in specific tissues (Figure 4). Gal4 is a protein that was originally identified in yeast which can activate genes by binding to an upstream activating sequence (UAS) located just upstream of the gene of interest (reviewed in Duffy 2002). Expression of the Gal4 protein is dictated by nearby enhancers, allowing for controlled expression of the driver. The UAS is linked to a gene of interest (GOI) but will only allow the GOI to be transcribed when bound to Gal4. Therefore, both the Gal4 and UAS components are required for expression of the GOI. This system allows for precise expression of a GOI: pairing the *Gal4* gene with tissue-specific enhancer sequences will cause the Gal4 to be expressed only in the tissues dictated by the enhancer. Because the UAS does not drive expression of the GOI without being bound to Gal4, the GOI is only expressed in the same tissues that the Gal4 is expressed in. Brand and Perrimon (1993) adapted this system for use in *D. melanogaster* by creating one line that contains a *Gal4* gene driven by an enhancer element and another that contains a GOI (such as a reporter) downstream of several UAS motifs. Crossing these two stocks together creates offspring that contain both *Gal4* and UAS motifs, therefore driving expression of the gene only in cells dictated

by the enhancer element. This method can be used to test the effect of a GOI in multiple tissues by using several Gal4 lines that utilize different enhancer elements.

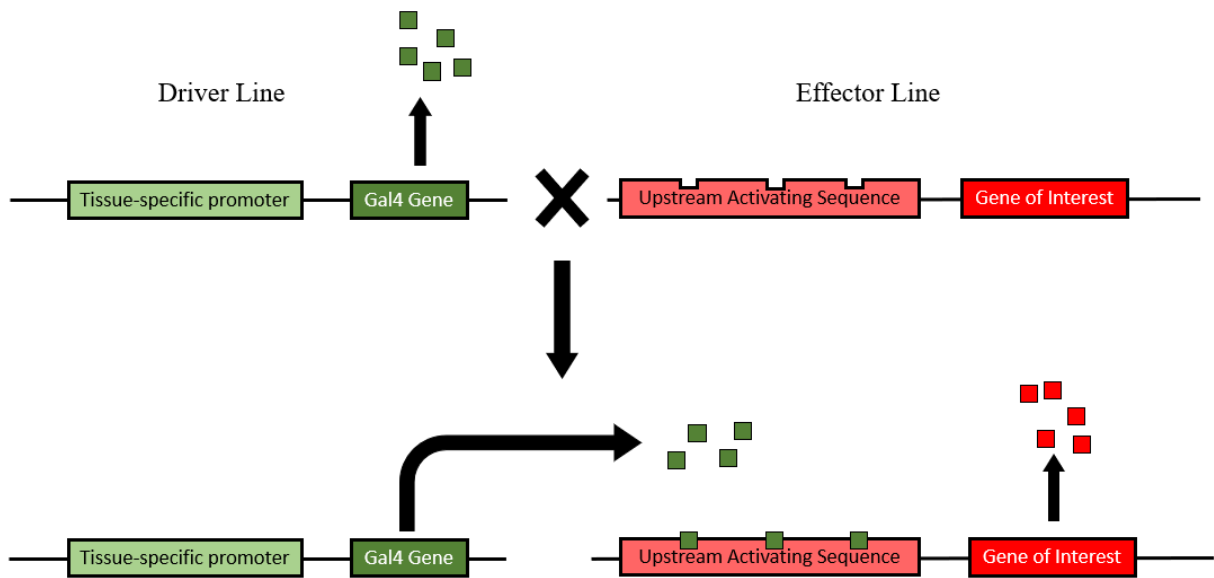


Figure 4: Driving expression of a gene in a particular tissue using the Gal4/UAS system.

The Gal4/UAS system functions by crossing a driver line, which contains only the *Gal4* gene paired with a tissue-specific promoter, to an effector line that contains the gene of interest (GOI) downstream of an upstream activating sequence (UAS). When the Gal4 protein binds to the UAS, it drives expression of the GOI. The GOI is not expressed in the parental lines because either the gene or driver is not present. The offspring of the cross contain both components however, so the GOI is expressed at high levels in the tissues dictated by the tissue-specific promoter.

Many *D. melanogaster* Gal4 lines have been produced and are readily available to drosophilists, allowing for tissue-specific expression of genes. The specificity of the Gal4/UAS system has recently been enhanced through the split Gal4/UAS system, which separates the DNA binding domain and activating domain of Gal4 and places both components under the control of different promoters. Gene activation produced by Gal4 binding to the UAS is only able to occur in cells where the promoter expression of the activating and DNA binding domains of Gal4 overlaps, allowing for extremely precise spatial control of gene expression (Luan et al. 2006). The resolution of the split Gal4/UAS system is such that gene expression can be limited to a single cell (Xie et al. 2018).

The pVALIUM20 vector combines RNAi with UAS, allowing it to be used with the Gal4/UAS system and express the shRNA constructs only in specific tissues. By designing shRNA that targets only *Kat60^{mel}* or *Kat60^{sim}* mRNA, it is possible to induce hemizygoty in hybrid females only in some neural regions to determine which subsets of neurons facilitate species-specific female rejection behaviour. One such candidate region is the mushroom body, as it is theorized to be a site of sensory integration and has also been implicated in courtship behaviour (Montague and Baker 2016; Yagi et al. 2016) If the candidate region does influence female receptivity to heterospecific males, then I expect that hybrids expressing shRNA specific to *Kat60^{mel}* will show reduced mating relative to hybrids expressing shRNA specific to *Kat60^{sim}* as there will be no *mel* katanin 60 to mask the *sim* rejection phenotype.

1.6 Summary of Experimental Objectives

During courtship, it is essential for organisms to be able to recognize conspecifics because of the heavy costs associated with forming interspecies hybrids. Many organisms use species-specific cues that are perceived and evaluated via neural pathways to recognize potential mates. The underlying genetic and neural basis of how these cues are processed into either receptive or rejection behaviour is almost entirely unknown. My objective was to determine whether *Kat60*, a gene involved in neuron development in *Drosophila* species, is involved in species identification during courtship.

The CRISPR/Cas9 system was utilized to knock out both a *mel* and *sim* allele of *Kat60* to produce hemizygous interspecies hybrids that are identical to wild-type hybrids at all loci except for *Kat60*. The mating behaviour of these hybrids can then be assessed using the reciprocal hemizygoty test (Stern 2014). If hybrids lacking *Kat60^{mel}* reject *mel* males significantly more often than reciprocal hybrids, then *Kat60* influences female rejection of heterospecific males. I also created stocks that silence either *Kat60^{mel}* or *Kat60^{sim}* in hybrids using allele-specific RNAi that is expressed in particular subsets of neurons via the Gal4/UAS system. Differences in behaviour would indicate that *Kat60* is involved in species recognition during courtship via that subset of neurons. If successful, this would be the first time that a gene has been linked to

interspecies mate rejection and this gene would provide the first insight into which neurons or brain regions contribute to that behaviour.

2 Materials and Methods

2.1 Fly Husbandry and Stocks

All *Drosophila* stocks were maintained in 30 mL vials containing standard cornmeal medium (Bloomington *Drosophila* Stock Center recipe) at 24 °C with 70% humidity and a 14h:10h light:dark cycle. A wildtype *D. simulans* line Florida City (FC) was obtained from Dr. Jerry Coyne and a wild-type *D. melanogaster* line BJS was obtained from Dr. Brent Sinclair. A *D. simulans* stock containing an inversion on the right arm of the third chromosome (*In(3R)Ubx, Ubxm/DII*) was obtained from Dr. David Stern. A *D. melanogaster* stock containing GFP-tagged sperm (w^* ; *TI{TI}Pkd2^{ko67}*, *P{protamineBeGFP2/CyO}*; *P{dj-GFP.S}3*) was obtained from Dr. John Belote. All other transgenic *D. melanogaster* stocks were obtained from the Bloomington *Drosophila* Stock Center (BDSC; Bloomington, Indiana). For creation of transgenic lines, a stock expressing phiC31 (Φ C31) integrase in the germ line and with an *AttP* site in the left arm of the third chromosome (Stock #25709: $y^1 v^1 P\{nos-phiC31\int.NLS\}X$; *P{CaryP}attP40*), a stock expressing Cas9 in the germ line (Stock #54591: $y^1 M\{nos-Cas9.P\}ZH-2A w^*$), and a stock containing multiple balancers (Stock #3703: $w^{1118}/Dp(1;Y)y^+$; *CyO/nub¹ b¹ sna^{Sco} lt¹ stw³; MKRS/TM6B, Tb¹*) was used. Multiple Gal4 *D. melanogaster* stocks will be used for the RNAi experiments: a stock driving Gal4 ubiquitously (Stock #3954: $y^1 w^1$; *P{w[+mC]=Act5C-GAL4}17bFO1/TM6B, Tb¹*), only in neural tissues (Stock #8760: w^* ; *P{GAL4-elav.L}3* and #458: *P{w[+mW.hs]=GawB}elav[C155]*), and only in the mushroom body (Stock #49265: w^{1118} ; *P{y[+t7.7] w[+mC]=GMR15E01-GAL4}attP2*).

2.2 Preparing CRISPR Constructs

2.2.1 Designing gRNA Sequences

Two different gRNA molecules were used to ensure complete gene knockout of *Kat60*. *Kat60* was previously sequenced in the two species strains I am using (Calhoun 2017), and sequences that are identical in the two species were located so that each gRNA can cut *Kat60* in either species. One gRNA molecule (5' GTA CTA GCT TTG TTA CGC GG) will target the 5'UTR of the gene while the other (5' GCG TAG GAA TGA CCG TAA TGG) will target the second exon.

These two cut sites were chosen because the Cas9 exonuclease will excise the start codon, exon 1, and part of exon 2, preventing functional product from being formed (Figure 5). While gRNA molecules are usually 20 nucleotides long (excluding the PAM), the two gRNA molecules that I designed are 17 or 18 nucleotides long. This was done to improve target specificity as gRNA molecules that are 17 or 18 nt long greatly reduce off-target mutagenesis while retaining their on-target functionality (Fu et al. 2014). Off-target mutagenesis was further prevented by comparing both gRNA sequences to the full *D. melanogaster* and *D. simulans* genome using NCBI BLAST (Altschul et al. 1990) to ensure that there were no off-target sites with at least 16 nt of complementarity.

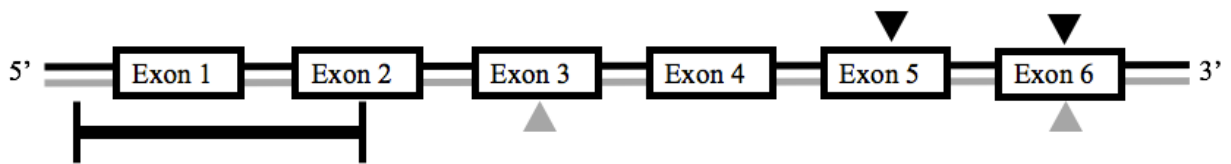


Figure 5: Schematic indicating where gRNAs and shRNAs bind to *Katanin 60*

The black line represents the *D. melanogaster* chromosome and the grey line represents the *D. simulans* chromosome. The bar represents the region excised by Cas9. Black triangles indicate where *D. melanogaster*-specific shRNA will bind, and grey triangles indicate where *D. simulans*-specific shRNA will bind.

2.2.2 Creating the gRNA Vector

The gRNA sequences were cloned into the pCFD4 vector (Port et al. 2014). The vector was a gift from Dr. Simon Bullock. pCFD4 allows for the expression of both gRNAs simultaneously from the same vector, reducing the number of components in the injection mix. pCFD4 also contains an *AttB* site which allows the vector to be integrated into flies containing an *AttP* site (Groth 2004) and a functional copy of the *vermillion* gene to find successful transformants (Fridell and Searles 1991).

My gRNA sequences were introduced into pCFD4 using a modified protocol outlined by Dr. Ben Ewen-Campen (<https://fgr.hms.harvard.edu/cloning-and-sequencing-0>). Oligos for PCR were ordered that contain the gRNA sequences between sequences complementary to pCFD4 (F: 5' tatataggaaagatatccgggtgaacttcGTACTAGCTTTGTTACGCGGgttttagagctagaatagcaag, R: 5'

attttaactgctatttctagctctaaaacCCATTACGGTCATTCTACGCgacgttaaattgaaaataggctc, uppercase letters denote gRNA sequences) and a PCR was run using pCFD4 as the template and the oligos as the primers. The PCR master mix consisted of 10 μ L of 5X Phusion HF Buffer, 5 μ L of 2 mM dNTPs mix, 2.5 μ L of a 10 μ M solution of each primer, 2 μ L of pCFD4 plasmid template, 0.5 μ L of Phusion HS Taq, and 27.5 μ L of UltraPure water. The PCR program is as follows: One cycle of 95 °C for 30 s; 36 cycles of 95 °C for 10 s, 61 °C for 30 s, and 72 °C for 1 minute; one cycle of 72 °C for 10 minutes. Phusion Taq Polymerase and its buffer were obtained from New England Biolabs (Ipswich, Massachusetts).

The pCFD4 vector was then digested with *Bbs*I (New England Biolabs) to linearize and remove the “filler” gRNA sequence present in the vector. The digestion mix was as follows: 2 μ g of pCFD4, 4 μ L of 10X NEB4 Buffer, 2 μ L of *Bbs*I, and UltraPure water up to 40 μ L. Fifteen microliters of PCR product and *Bbs*I-digested backbone were then run on a 1% TBE agarose gel and the appropriate bands were cut out and extracted using a Geneaid purification kit (FroggaBio). The PCR product was then ligated into the digested pCFD4 using a Gibson assembly kit (New England Biolabs) according to this recipe: 4 μ L of digested pCFD4, 1 μ L of PCR product, and 5 μ L of the Gibson Assembly mix. The mixture was incubated at 50 °C for 1 hour, and then transformed into NEB 10-beta competent *E. coli* cells (New England Biolabs) using a 30 second heat shock at 42 °C and a 1 hour incubation in SOC medium at 37 °C. Twenty microliters of the reaction were plated onto LB+Ampicillin plates and incubated at 37 °C overnight. Four colonies per reaction were selected and grown in 1 mL of LB+Ampicillin (100 μ L/mL) for 24 hours at 37 °C. Plasmid DNA was extracted using a QIAprep spin miniprep kit (Qiagen, Hilden, Germany) and sent to Roberts DNA Sequencing Facility (London, ON) for sequencing using the primer 5' GAC ACA GCG CGT ACG TCC TTC G.

2.3 Preparing shRNA Constructs

2.3.1 shRNA Design

Allele-specific shRNA constructs were created by aligning *D. melanogaster* and *D. simulans* *Kat60* gene sequences and finding regions with high concentrations of mismatches as this

decreases the likelihood of silencing the wrong allele. All potential shRNA sequences that shared 19 or more nucleotides with any other gene or mRNA product in either species were discarded due to potential off-target effects. Two constructs for each species were designed to increase the likelihood of silencing the desired *Kat60* allele. The *D. melanogaster* shRNA constructs target exon 5 and 6 whereas the *D. simulans* constructs target exon 3 and exon 6 (Figure 5). All Gal4 lines were sequenced (F: 5' ATC GAC GAG ATC GAC TCC CT, R: 5' GTA CTT GTC CAG GTC TGC CC) to confirm that all *mel*-targeting shRNA sequences match the *Kat60* sequences in each Gal4 line.

2.3.2 Creating the shRNA Vector

All shRNA constructs were cloned into the vector pVALIUM20, which was a gift from Dr. Norbert Perrimon and Dr. Jian-Quan Ni. The vector contains several upstream activating sequences (UAS) paired to the heat-sensitive promoter *Hsp70* (Ni et al. 2011). This allows for time- and tissue-specific expression of the shRNA: hairpin expression can be controlled temporally by exposing the flies to high temperatures (28 °C) at the desired developmental stage and spatially by utilizing tissue-specific Gal4s (Ni et al. 2007). Like pCFD4, pVALIUM20 contains an *AttB* site which allows for site-specific integration of the vector using Φ C31 integrase (Groth 2004).

All four of my desired shRNA sequences were cloned into pVALIUM20 using a slight modification of the protocol from Chang et al. (2014). The hairpin was constructed by adding “tagttatattcaagcata” to the 5' end of the antisense strand and then adding the complement of the antisense sequence to the 5' end of that oligo. This is known as the “top” strand. The complementary sequence of the “top” strand was then generated to form the “bottom” strand. CTAGCAGT and GCG were added to the 5' and 3' end of the “top” strand respectively, and AATTCGC and ACTG were added to the 5' and 3' end of the “bottom” strand respectively. These additions create the *NheI* and *EcoRI* sites used to integrate the hairpin construct into pVALIUM20. The sequences of all oligos that were used create the four different pVALIUM20 constructs can be found in Figure 6.

Oligos were diluted to a concentration of 20 μ M and 10 μ L of both the “top” and “bottom” oligo were mixed with 100 μ L of 1.2X annealing buffer (12 mM pH 7.5 Tris-HCl, 120 mM NaCl, and 1.2 mM EDTA). This mixture was then heated to 95 °C for 5 minutes and then cooled to room temperature. Next, pVALIUM20 plasmid was linearized with *NheI* and *EcoRI* (New England Biolabs). The double-digest protocol was as follows: 1 μ L of each restriction enzyme, 10 μ L of pVALIUM20 DNA, 5 μ L of NEBuffer1.1, and 33 μ L of UltraPure water were mixed and incubated at 37 °C for 4 hours followed by 20 minutes at 65 °C to heat-inactivate the enzymes. The annealed oligos were then ligated into the linearized pVALIUM20 by mixing 6 μ L of the annealed oligo product with 1 μ L of linearized pVALIUM20, 10 μ L of 2X ligation buffer, 1 μ L of T4 DNA Ligase, and 2 μ L of UltraPure water and incubating the mixture at 16 °C for two hours.

The ligated pVALIUM20 product was added to NEB 10-beta competent *E. coli* cells (New England Biolabs) which were then transformed using a 30 second heat shock at 42 °C and a 1 hour incubation in SOC medium at 37 °C. Twenty microliters of this media were plated onto LB+ampicillin plates (100 μ L/mL) and grown overnight at 37 °C. Four colonies were then selected and grown in 1 mL of LB+Ampicillin (100 μ L/mL) for 24 hours and plasmid DNA was extracted using a QIAprep spin miniprep kit (Qiagen). A PCR was then run on the extracted plasmid DNA to verify successful transformation (forward primer: 5' ACC AGC AAC CAA GTA AAT CAA C, reverse primer: 5' TAA TCG TGT GTG ATG CCT ACC). The PCR program consisted of: 95 °C for 5 mins; 35 cycles of 95 °C for 30s, 52 °C for 30s, and 72 °C for 1 min; and 72 °C for 10 min. The PCR product was run on a 1% TBE agarose gel. Successful transformants were marked by a 350 bp PCR product, whereas unsuccessful colonies gave a 900bp product (Figure 7). All 350 bp PCR products were sent to Robarts DNA Sequencing Facility (London, ON) for verification using the forward primer.

Exon 3

<i>melanogaster</i>	A	A	C	G	T	C	C	G	C	C	A	C	A	G	C	A	C	C	G	A	G
<i>simulans</i>	A	G	C	G	T	C	C	G	C	C	G	C	A	G	C	A	C	A	G	A	G

Exon 5

<i>melanogaster</i>	G	A	G	T	G	A	A	G	T	C	T	G	A	G	C	T	G	C	T	G	G
<i>simulans</i>	G	T	G	T	G	A	A	G	T	C	G	G	A	G	T	T	G	C	T	G	G

Exon 6

<i>melanogaster</i>	G	G	A	C	T	G	A	C	C	C	A	G	A	G	C	A	G	A	T	T	
<i>simulans</i>	G	G	G	C	T	G	A	C	T	C	C	A	G	A	G	C	A	G	A	T	T

Figure 6: Differences between alleles in regions used to create shRNA constructs.

Four shRNA constructs were designed to target *Kat60* in a species-specific manner. Exon number and species name indicates which exon and allele of *Kat60* is bound by that construct. Shaded nucleotides indicate differences between alleles. Sequences that are shaded grey are shown for comparison but were not used to create shRNA.

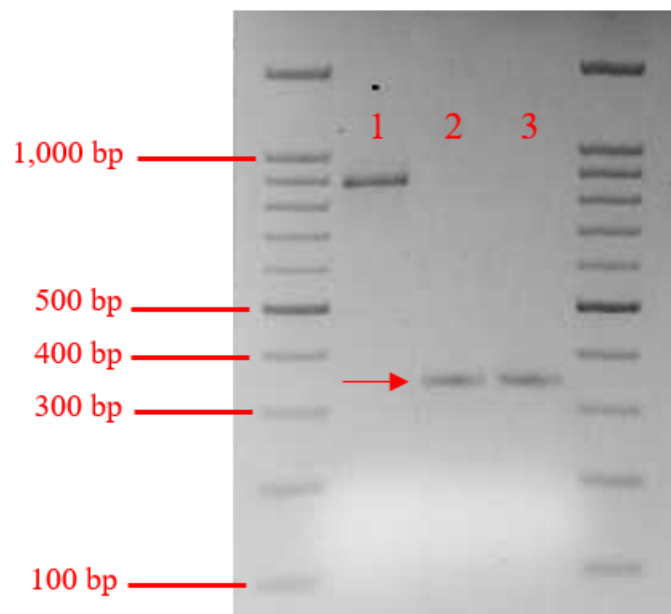


Figure 7: Screening for colonies containing ligated pVALIUM20

Vectors were screened with PCR to confirm that the cells were transformed with the correct vector. Bands amplified from vectors that contain the shRNA sequence are 350bp long whereas bands from vectors that do not contain shRNA are 900 bp long. The arrow indicates bands from colonies containing shRNA in the vector (lanes 2 and 3). Sample DNA was electrophoresed alongside a 100 bp DNA ladder.

2.4 Microinjection Protocol

2.4.1 Microinjection Preparations

Approximately 500 flies were transferred to a fly cage with an apple juice agar plate topped with active yeast paste 2–7 days before injecting and kept in a 25 °C incubator. Apple juice plates were replaced at least once a day. Loading needles were prepared by stretching a 1.0mm borosilicate tube over a Bunsen burner by hand and breaking it in the centre. Injection needles were pulled on a Micropipette Puller P-97 needle puller (Sutter Instrument Company), provided by Dr. Greg Gloor. Most injection mixes were prepared in advance by mixing a 10µL aliquot of the desired vector with 2µL of blue food colouring and storing at -20 °C until use. The *D. simulans* CRISPR injection mix was prepared immediately before use by mixing 1µL of Cas9, 1.5µL of pCFD4, 2.5µL of Cas9 Buffer (NEB), and 1µL of blue food dye because the Cas9 protein loses functionality after repeated freeze-thaw cycles. The Cas9 protein was a generous gift from Dr. David Edgell.

2.4.2 Microinjection Protocol

Apple juice agar plates topped with active yeast paste were changed 30 minutes before injections began in order to remove old embryos. New fly embryos were washed off the apple juice plate with distilled water and collected in baskets made of 1.5” Sheer Ribbon. Embryos were washed with distilled water in the basket to remove yeast paste and aligned on a coverslip. Embryos were coated in extra-virgin olive oil immediately prior to injection and visualized using a dissecting microscope (Nikon Stereo microscope). Embryos were injected at the posterior end and the oil was washed off immediately after injection.

2.5 Post-Injection Care, Screening, and Crosses

The injected embryos were placed in a petri dish filled with food and kept in a 24 °C incubator with a 14:10 light:dark cycle until pupation. Each pupa was transferred from the petri dish to its own food vial.

2.5.1 Screening and Balancing RNAi Lines

Injected *D. melanogaster* embryos that reached adulthood were crossed to other adults who were injected with the same construct. This was done to increase the likelihood that the F1 generation would contain the construct. Adults were crossed to the parental stock (#25709) when no injected adults of the opposite sex were available. Transgenic offspring were identified by their wild-type (rather than *vermillion*) eyes (Figure 8; Figure 9). Virgin transgenic females were crossed to the balancer stock (#3703) to maintain and track the transgene during further crosses. A full schematic of the RNAi crossing scheme is provided in Figure 9. The crosses produced two different transgenic lines: one that contained a balanced transgene on chromosome 2 and a balanced Gal4 on chromosome 3, and one that contained a balanced transgene on chromosome 2 and two different chromosome 3 balancers. The first line was used to produce hybrids for mating assays whereas the second line was crossed to other Gal4 stocks. Efficacy of the Gal4 stocks was confirmed by crossing them to $w^1, y^1; UAS-GFP$ flies, dissecting the brains of offspring, and seeing GFP excitation in the correct brain regions of F₁ flies under 488nm wavelength light.

2.5.2 Determining Functionality of shRNA Constructs

Constructs targeting *mel Kat60* were tested by extracting RNA from ten pure-species third-instar larvae, whereas constructs targeting *sim Kat60* were tested by extracting RNA from hybrid adults that contain both the Gal4 and shRNA construct. Hybrid adults needed to be used as the dominant marker on the balancers are not visible in larvae. Flies and larvae were heat-shocked at 30 °C for two hours and homogenized in 500 µL of Trizol, then 100 µL of chloroform was added. The mixture was shaken vigorously for 30 seconds and incubated at room temperature for 5 minutes. The aqueous phase was separated from the organic phase by centrifuging at 13,000 RCF for 15 minutes and transferred to a new tube. An equal volume of 70% RNase-free ethanol was added to the aqueous phase and vortexed to mix. The sample was then transferred to a PureLink Mini Kit (ThermoFisher, Waltham, Massachusetts) spin column and RNA was extracted by following the provided protocol. Extracted RNA concentration and quality was determined using a spectrophotometer. cDNA of the extracted RNA was produced using a slightly modified Maxima™ H Minus cDNA Synthesis Master Mix with dsDNase kit (ThermoFisher). The total volume of the reaction was doubled to 20 µL to increase the amount

of usable product and 2 μg of RNA was used in each reaction. RT-PCR was performed using the primer set F: 5' AAC GTC CGC CAC AGC ACC GAG, R: 5' CCA GCA GCT CAG ACT TCA CTC and a thermocycler protocol that was as follows: 95 °C for 3 minutes; 25 cycles of 95 °C for 30 seconds, 56.8 °C for 10 seconds, and 72 °C for 30 seconds; and 72 °C for 3 minutes. Five microlitres of the PCR product was run on a 1% agarose-TBE gel to visualize the results.



Figure 8: Identifying transgenic offspring

Transgenic offspring can be recognized by their wild type eyes. The male (top) does not contain the transgene and has bright orange eyes indicative of *vermillion* mutants. The female (bottom) has the transgene and darker wild-type eyes indicating *vermillion* rescue. Both flies were collected ~6 hours after eclosion and were produced by the same screening cross.

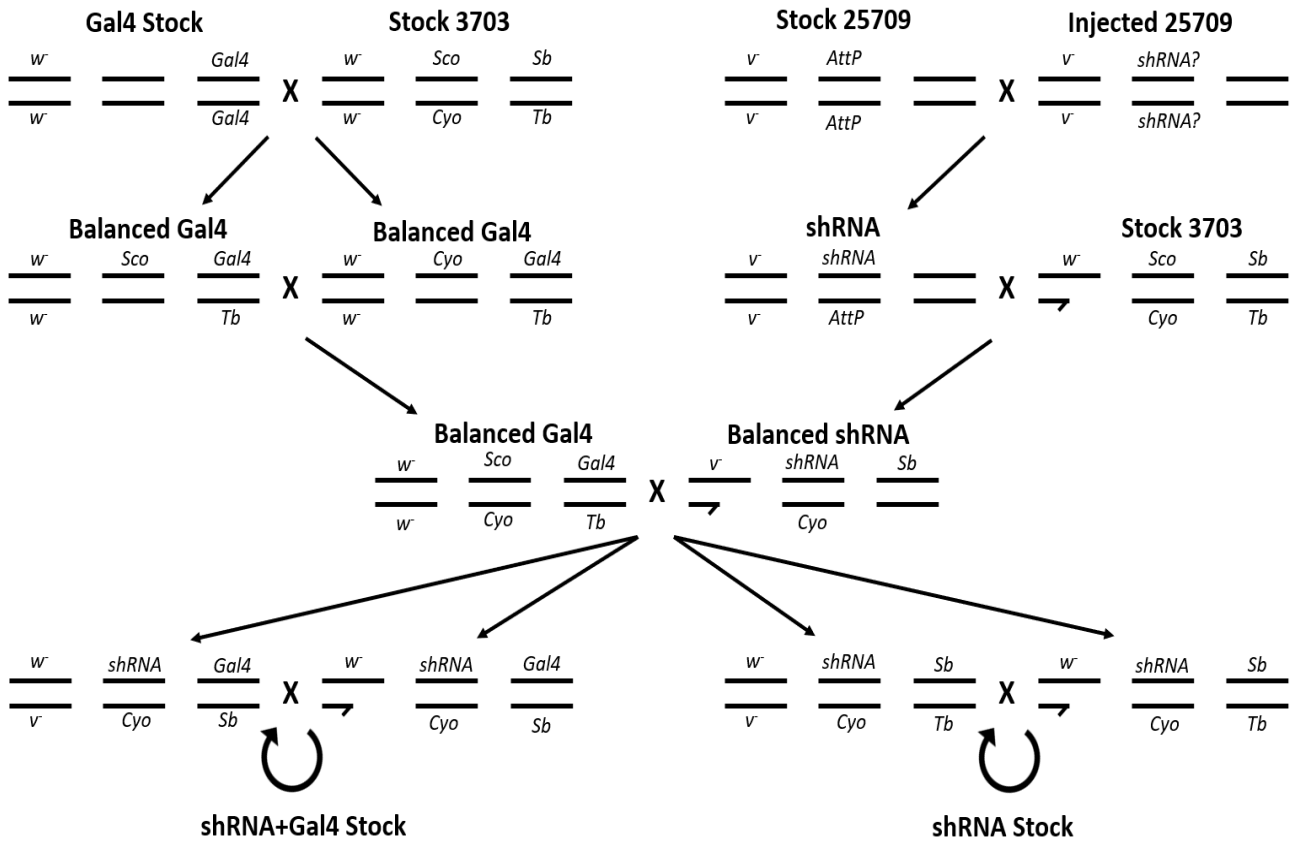


Figure 9: Crossing scheme used to produce transgenic shRNA stocks

Horizontal bars represent chromosomes and forked bars represent Y chromosomes. Male genotypes are only shown in crosses that require males and females of a specific genotype, otherwise males and females are interchangeable. These crosses produce two stable stocks: one that contains a balanced insert and two third chromosome balancers (shRNA stock), and one that contains a balanced insert and a balanced Gal4 (shRNA+Gal4 stock). The shRNA+Gal4 stock can be used for mating assays whereas the shRNA stock can be easily crossed to other Gal4 lines to produce additional shRNA+Gal4 stocks.

2.5.3 Screening and Balancing CRISPR Lines

Injected *D. melanogaster* flies that reached adulthood were screened the same way as described for RNAi. Balanced transgenic males were then crossed to a line producing Cas9 in the germline (y^1 *M{nos-Cas9.P}{ZH-2A w**). Offspring produced from that cross were crossed with to the balancer stock (#3703) until larvae were visible, at which point the father had his DNA extracted and PCR-screened to detect deletions in *Kat60*. The primers used to screen for deletions were F: 5' CCA GAG CAC GCA CAT CAC and R: 5' GTC GTA GTT GCC CGT CAG AG and the PCR program used to test for deletions was as follows: 95 °C for 5 mins; 35 cycles of 95 °C for 30s, 51 °C for 30s, and 72 °C for 2 min; 72 °C for 10 mins. PCR product was run on a 1% TBE agarose gel at 130V for 90 minutes to visualize band size. Unmodified *Kat60* yields a band ~1.1kbp long, whereas a full deletion from both gRNA target sites produces a band ~200bp long. Siblings produced from fathers containing a *Kat60* deletion were mated to each other until larvae were visible, at which point both parents were PCR screened for *Kat60* deletions. Offspring produced from crosses where both parents contain a deletion in *Kat60* were used to produce a stable *Kat60*^(mel-) deletion stock that can be used in mating assays. The full *D. melanogaster* CRISPR crossing scheme is given in Figure 10a.

Injected *D. simulans* adults were crossed to the *D. simulans* inversion line. Injected parents were screened for *Kat60* deletions using the same method outlined above. If the deletion was present, their offspring were mated brother-to-sister and screened for the deletion. Both parents were screened once offspring from that cross are visible. The offspring of parents who both contained the deletion made a stable *Kat60*^{sim-} stock that was used in mating assays. The crossing scheme for *D. simulans* CRISPR is given in Figure 10b.

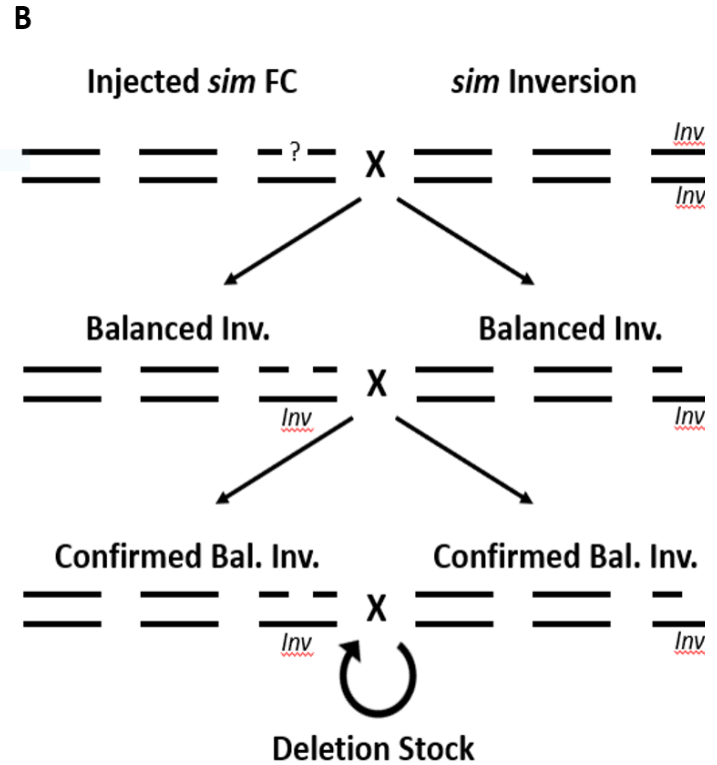
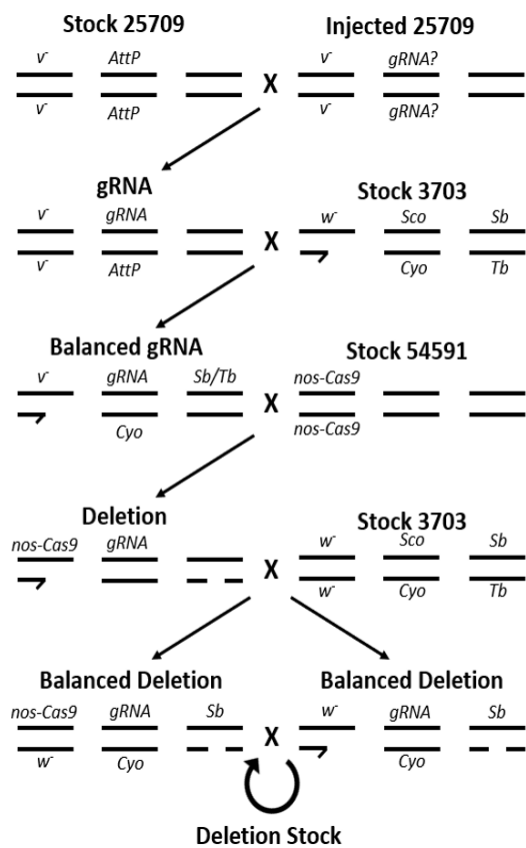


Figure 10: Crossing scheme to create *Katanin 60* deletion stocks

Horizontal bars represent chromosomes, forked bars represent the Y chromosome, and gaps represent deletions. **A)** CRISPR in *D. melanogaster*. After producing offspring, flies of the “Deletion” genotype are screened using PCR to confirm the presence of a deletion in *Kat60*. If a deletion is present, that fly’s offspring are mated brother-to-sister and PCR screened to identify crosses where both parents contain the deletion. The offspring of two such parents become a stable deletion stock for *Kat60*^(mel-). **B)** CRISPR in *D. simulans*. Injected flies that reach adulthood are crossed to *D. simulans* flies containing an inversion on the third chromosome. After producing offspring, the injected parents are screened using PCR to confirm the presence of a deletion in *Kat60*. If a deletion is present, offspring are crossed brother-to-sister and are themselves screened once their offspring are visible. The offspring of two parents that both contain a balanced deletion become a stable stock for *Kat60*^(sim-).

2.6 Behavioural Assays

2.6.1 Test Crosses

Female *D. melanogaster* virgins from each shRNA; Gal4 stock were collected 0–8 hours after eclosion, transferred to new food vials under CO₂ anaesthesia, and aged 5–7 days to ensure reproductive maturity and virginity. Female virgins were also collected from the corresponding Gal4 stock and *D. melanogaster* strain BJS and stored in the same way. F₁ female hybrids were produced by crossing 8–10 female virgins to 20–25 *D. simulans* males in a food vial with the cotton plug pushed down. The interspecies hybrid crosses took place in a 28 °C incubator set to a 14:10 hour light cycle. These crosses produced four *sim/mel* hybrid genotypes to be tested: (*sim/RNAi*; *sim/Gal4*), (*sim/RNAi*; *sim/Bal*), (*sim/mel*; *sim/Gal4*), and (*sim/mel*; *sim/mel*).

Female *D. melanogaster* virgins from each *Kat60* deletion stock were collected and crossed in the same manner but placed in a 24 °C incubator set to a 14:10 hour light cycle. The *Kat60* deletion stock was also crossed to *D. melanogaster* strain BJS to control for potential effects of the deletion on intraspecific mating behaviour. The inter- and intraspecies crosses each produced two genotypes for testing: *sim/Kat60^{Del}* and *sim/Bal* interspecies hybrid females and *mel/Kat60^{Del}* and *mel/Bal* intraspecies females.

2.6.2 Mating Assays

Females were collected 0–8 hours after eclosion and sorted based on the presence or absence of the dominant markers corresponding to balancer chromosomes under CO₂ anaesthesia. Virgin females were transferred to new food vials with each vial containing up to 10 females. Females were assayed 5–7 days after eclosion. *D. melanogaster* males with GFP-tagged sperm were collected and maintained the same way. Assays were performed by putting a virgin female and male in the same 30mL food vial and observing the courtship and mating behavior of both flies for 45 minutes. Equal numbers of each test genotype were tested simultaneously to control for environmental effects. Assays testing the effect of RNAi knockdown of *Kat60* were carried out at 28 °C with 70% relative humidity whereas assays testing the effect of *Kat60* deletions would be carried out at 24 °C with 70% relative humidity. Females and males were kept together for 24

hours, after which the female reproductive tract was dissected to determine the presence or absence of sperm. Sperm presence was used as a proxy for mating as many interspecies mating events would not occur within the relatively short 45-minute observation period. The proportion of pairs that mated would be analyzed using a G-test. Significant ($p < 0.05$) reduction in female receptivity after deletion or RNAi knockdown of the *D. melanogaster* copy of *Kat60* would indicate that this gene influences female mating behavior.

3 Results

3.1 Determining Functionality of Gal4 Stocks

I tested the functionality of four Gal4 stocks by crossing them to a UAS-GFP line and imaging the brains. At least three brains per genotype were dissected. The Gal4 stocks #3954 (*Act5C-Gal4*, Figure 11A) and #6920 (*elav-Gal4*, Figure 11B) initially showed the expected pattern of GFP expression. Stock #8760 (*elav-Gal4*, Figure 11C) did not ubiquitously express GFP in all parts of the brain. For example, there was no GFP fluorescence visible in the optic lobes. The version of stock #49265 (*rut-Gal4*, Figure 11D) that was given to me by a previous lab member had lost its functionality, as it was no longer driving Gal4 in any of the mushroom body lobes. GFP expression was observed in the mushroom body lobes after reordering it, however (Figure 11E).

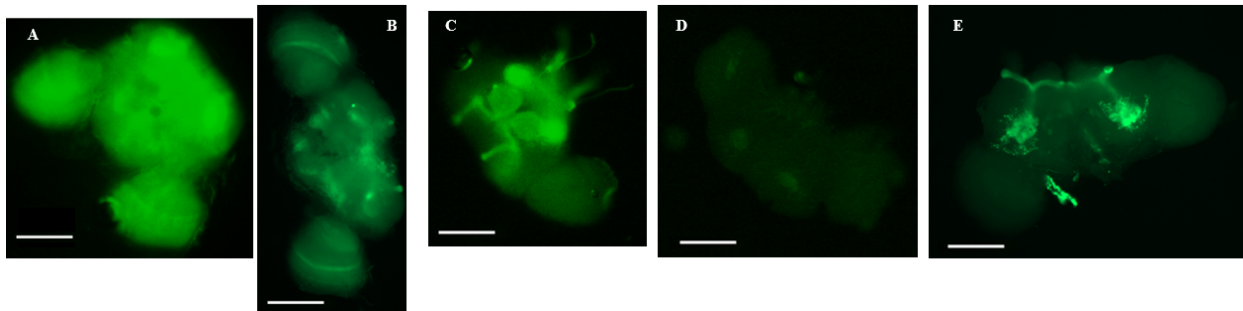


Figure 11: Functionality of the Gal4 Stocks

Images of *D. melanogaster* brains to test the functionality of the (A) *Act-5C-Gal4* (stock #3954), (B) *elav-Gal4* (#6920), (C) *elav-Gal4* (#8760), (D) old *rut-Gal4* (#49265), and (E) new *rut-Gal4* (#49265) stocks when paired with UAS-GFP. Note that the functionality of stock #8760 and old #49265 did not show the expected pattern of Gal4 expression. Scale bars represent 100 μm .

3.2 Creating *Kat60*-targeting Vectors

All four shRNA constructs and the gRNA construct were successfully cloned into their respective vector. The shRNA vectors were checked for accuracy using PCR (Figure 7, Figure 12A) and all constructs were verified by sequencing (Figure 12B, C).

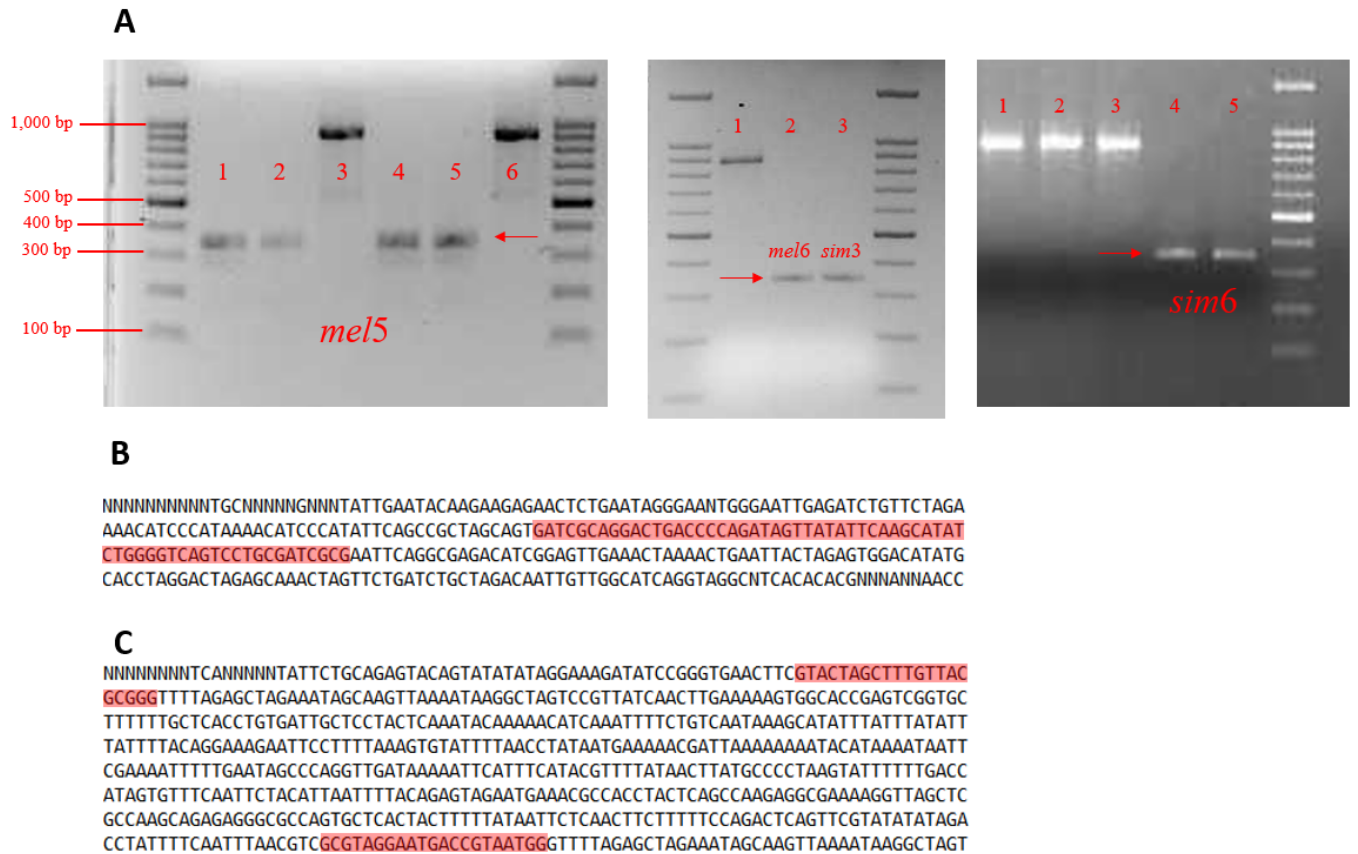


Figure 12: Confirming the accuracy of all *Kat60*-targeting vectors.

A) pVALIUM20 vectors that contain the shRNA of interest produce a band ~350bp long (indicated by an arrow), whereas untransformed vectors produce a band ~900bp long. Confirmation of *mel*-Exon5 is shown in the left image (lanes 1, 2, 4, and 5), *mel*-Exon6 and *sim*-Exon3 are confirmed in the middle image (lanes 2 and 3 respectively), and *sim*-Exon6 is confirmed in the right image (lanes 4 and 5). Sample DNA was electrophoresed alongside 100 bp DNA ladder **B)** Example of sequencing data that confirm successful cloning of an shRNA sequence into pVALIUM20. The data were used to confirm the sequence of *mel*-Exon6. The shRNA sequence is indicated by the shaded region. **C)** Example of sequencing data that confirms successful cloning of my gRNA sequence into pCFD4. The gRNA sequences are indicated by the shaded region.

3.3 Embryo Survival After Microinjection

Efficiency of transgenesis is highly dependent on both the number of embryos that can be injected in 30 minutes and the survivability of embryos post-microinjection. Practice increases both rate of injection and embryo survival. For simplicity, I will refer to each 30-minute period spent microinjecting as a “round” of injections whereas I will refer to the cumulative number of embryos injected that day as a “session”. I initially could inject 30 embryos per round, but increased to my maximum, 119 injections per round, after 3 months of practice. It took 37 sessions (~5 months) of microinjections using an optimized protocol to achieve at least a 10% survival rate consistently (Figure 13). I was able to produce several of my transgenic stocks during my initial rounds of microinjections however, which indicates that vector integration using Φ C31 integrase is highly robust and can be effective even in the hands of inexperienced researchers. Integration efficiency could not be determined using my crossing scheme as injected survivors were crossed with each other, which made it difficult to determine how many parents successfully had the vector integrated into the germline. I injected 4,875 embryos in total, 489 of which reached adulthood for an overall 10.03% post-injection survivability rate.

Post-microinjection Survivability

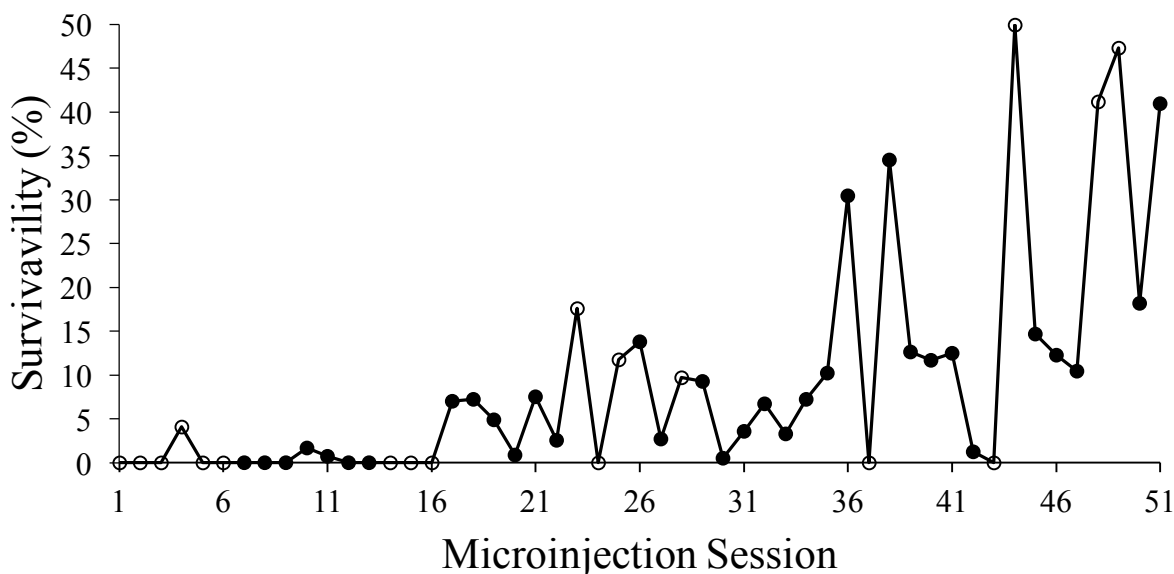


Figure 13: Change in post-microinjection survival of embryos over time.

Embryo survivability post-injection was calculated by counting the number of embryos that produced adult flies and dividing that by the total number of embryos injected that session. All sessions that consisted of fewer than 50 injections are marked using unfilled circles as they could skew results.

3.4 Creating Transgenic shRNA Lines

I have produced 17 different successful transgenic fly insertions for this project in total, 16 of which are with/of shRNA (Table 1). Four of these insertions have been made into balanced shRNA lines which can be used to easily create new shRNA-Gal4 line combinations (Figure 9, bottom right). Twelve transgenic insertions have been made into both the balanced shRNA line and a shRNA-Gal4 line by crossing three Gal4 stocks (#3954, #8760, and #49265) into each of the four balanced shRNA lines (Figure 9, bottom left). Finally, I have produced a balanced gRNA line to induce deletions in the gene sequence of *Kat60*. The shRNA-Gal4 lines with stock #6920 are currently in progress as that line was obtained much more recently than the others.

Table 1: Time to completion of all shRNA transgenic lines.

	<i>sim-exon3</i>	<i>sim-exon5</i>	<i>mel-exon5</i>	<i>mel-exon6</i>
Balanced Transgene	Complete	Complete	Complete	Complete
49265 (<i>rut-Gal4</i>)	Complete	Complete	Complete	Complete
8760 (<i>elav-Gal4</i>)	Complete	Complete	Complete	Complete
6920 (<i>elav-Gal4</i>)	3 weeks	3 weeks	3 weeks	3 weeks
3954 (<i>Act5C-Gal4</i>)	Complete	Complete	Complete	Complete

3.5 Knocking Out *Kat60* with the CRISPR/Cas9 System

I used the CRISPR/Cas9 system to create the expected ~900bp deletion in *mel Kat60* (Figure 14). Each of the four flies arising from an injected embryo contained at least some cells with the full deletion, indicating that the protocol used in *mel* to induce deletions worked. I have not produced any *sim* flies that contain a similar deletion yet, but the results in *mel* are encouraging and proof that the construct and gRNA function as predicted. Generation of a stable stock bearing the deletion is in progress, as is confirming the breakpoints of the deletion through sequencing.

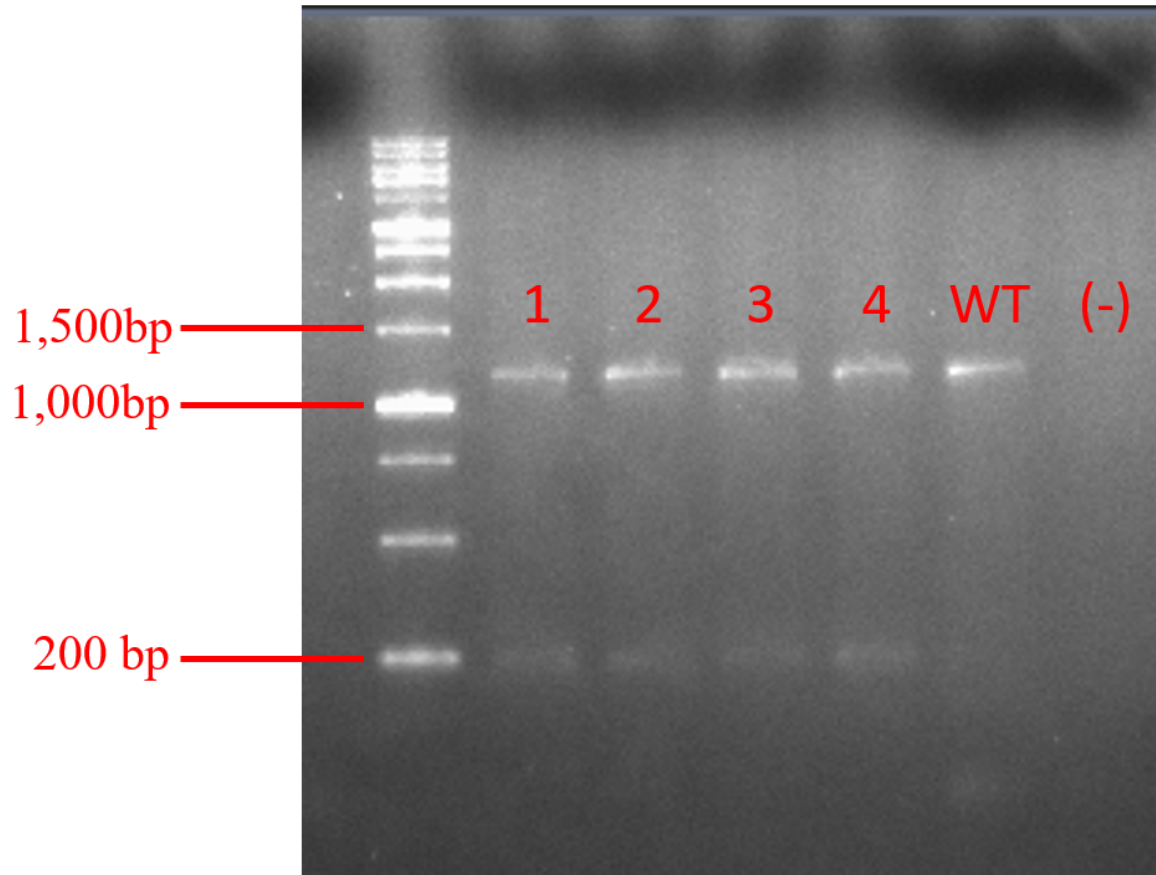


Figure 14: Inducing deletions in *D. melanogaster Kat60* using the CRISPR/Cas9 system.

PCR was run on DNA from offspring of parents containing pCFD4 or *nanos-Cas9* (see Figure 10) to determine if they contained a CRISPR-induced deletion. Flies containing wild-type *Kat60* only produce a band corresponding to a DNA fragment 1,100bp long (WT lane) whereas injected flies containing mutated *Kat60* produce two bands, one corresponding to a DNA fragment 1,100bp long and one 200bp long (lanes 1–4). The faint band at the bottom of the WT lane is unused primer. Sample DNA was run alongside a 1 kb DNA ladder.

4 Discussion

I successfully produced multiple transgenic *D. melanogaster* lines that can be used to determine *Kat60*'s effect on female receptivity towards heterospecific males. My ability to create these lines relied on efficient microinjections and transgene design that maximized construct potency while maintaining specificity. I was unable to gather any mating assay data due to lethality problems, but the lines I created can be used immediately after these issues are resolved.

4.1 Optimizing and Improving Microinjections

Because both aspects of my project (CRISPR and RNAi) relied on my ability to microinject embryos, I optimized the microinjection protocol to improve efficiency and survival. Time is extremely limited when injecting, as embryos must be injected before the formation of pole cells (Cartwright 2009), otherwise the injection mix cannot reach the nuclei and generate germline mutations after nuclei are encased by a cell membrane. In *D. melanogaster*, pole cells form after the tenth cell division and eventually give rise to germ cells (Illmensee 1973). This occurs roughly two hours after the egg is fertilized (reviewed in Gilbert 2000). Most microinjection protocols call for 30 minutes of egg laying and 30 minutes of microinjecting (Bachmann and Knust 2008), and the number of eggs that can be injected in this time is influenced by the amount of time spent preparing the embryos for injection. *Drosophila melanogaster* embryos can be injected with or without their chorion (the eggshell), and both methods have benefits and drawbacks. Removing the chorion allows for easier visualization of the injection mix within the embryo which increases precision and can also prevent needles from clogging or breaking against the chorion. However, removing the chorion can be a slow process without specialized filtration equipment (Cartwright 2009), as it can take 3–4 minutes to dechorionate and wash all the embryos. Dechoronation is also usually performed by washing the embryos in bleach and a previous member of my lab found that this process reduced survival by over 50% (Bielaska Da-Silva 2017). Because of these disadvantages, I elected to inject embryos with the chorion on, which increased both the number of embryos I could inject and their survival rate.

I integrated all my constructs into *D. melanogaster* by using this protocol. It has the further advantage of being relatively easy to learn, as other members of my lab could integrate their own constructs despite having less practice with the technique. Unsurprisingly, the number of embryos I could align per round and post-injection survivability increased as I became more experienced with the protocol. It took 23 microinjection sessions to reach 10% post-injection survival, but I could consistently maintain 10% survival after 39 sessions (Figure 13). I reached my maximum number of embryos aligned per round during session 29, roughly three months after I began using this protocol. This protocol can be used for all techniques that require germline transformations in *Drosophila*, such as *P*-element transformations (Venken and Bellen 2007) or *Minos* insertions (Metaxakis et al. 2005).

4.2 Optimizing Transgenesis

4.2.1 Optimizing Gene Silencing

Many factors should be considered when designing shRNA as small changes in hairpin sequence can affect specificity and efficacy of the construct. For example, Vert et al. (2006) found that siRNA guide strands that began with a uridine are particularly potent. This was also found to be the case in shRNA (Knott et al. 2014). This increase in potency is likely because the first nucleotide of the guide strand is incorporated into RISC rather than being used for binding to the target mRNA (Elkayam et al. 2012; Nakanishi et al. 2012). Future shRNA designs could be improved by making the 5' nucleotide of the guide strand uridine regardless of complementarity to improve loading into RISC. If possible, it would also be ideal to target low GC regions of the gene of interest as low GC content was found to be predictive of high shRNA efficacy (Knott et al. 2014). The requirements of my project prevented me from selecting regions of *Kat60* at my discretion as the shRNA I designed needed to be able to differentiate between the *sim* and *mel* alleles of *Kat60*. Creating allele-specific shRNA forced me to select regions with the greatest number of mismatches between the two alleles. Complicating this issue is the fact that *Kat60* is nearly identical between these two species (Figure 6) (Calhoun 2017), making it difficult to find regions that contain at least two mismatches in a 21-nucleotide range. This is also why I needed

to develop my own RNAi constructs instead of ordering *Kat60*-targeting RNAi stocks already available at the Bloomington *Drosophila* Stock Center.

While integrating pVALIUM20 and creating shRNA stocks was relatively simple, maintaining some of the stocks was difficult because *Kat60* is an essential gene. My *mel*-exon5; *Actin5C-Gal4* stock has been particularly difficult to maintain. This is likely because pVALIUM20 is still transcribed at 24 °C (Ni et al. 2011), causing the *Actin5C* enhancer to drive Gal4 expression in all tissues. High levels of Gal4 expression could further amplify any leaky expression of the *mel*-targeting shRNA. If expression levels of the shRNA are high enough the shRNA could cause lethality as it would significantly reduce or eliminate expression of this essential gene. This effect can be reduced by rearing the stock at lower temperatures, but Ni et al. (2011) also found that pVALIUM20 is able to induce strong gene silencing even at 18 °C. As 18 °C is near the lowest temperature that *D. melanogaster* can tolerate before experiencing a reduction in longevity (Cohet 1975), it is possible that there will always be some shRNA expression. I also noticed that the *Actin5C-Gal4* transgene itself also affects fly viability as a single copy of the transgene caused some lethality and it was impossible to create flies homozygous for the *Ga4* transgene. Since the *mel*-exon5 shRNA construct does not noticeably alter viability when paired with other broadly-expressed Gal4s (such as *elav-Gal4*), it is likely that the low viability of this stock is due to this combination of driver and shRNA construct. One way to bypass this problem would be to reduce how responsive the vector is to Gal4 binding. pVALIUM20 was designed with two 5x UAS motifs to drive expression of the shRNA, but one of those motifs can be removed using the Cre-*LoxP* system. This modification might be worthwhile if rearing at lower temperatures is not sufficient to rescue stocks that are difficult to maintain.

4.2.2 Optimizing CRISPR Efficiency

Maximizing target specificity is important when designing both gRNA and shRNA. The most obvious way to maximize the specificity of gRNA molecules is to change the 20-nucleotide sequence that is complementary to the target sequence. A study that examined the effect of a single mismatch on off-target effects found that mismatches distal to the PAM were less effective at preventing off-target binding (Morgens et al. 2017). Therefore, gRNA molecules

should be designed to have mismatches with likely off-targets as close to the PAM as possible. Off-target effects can be further reduced by using truncated gRNAs, which have guides 17–18 nucleotides long rather than the usual 20 (Fu et al. 2014). Truncated gRNA molecules also do not require mismatches to be near the PAM to be most effective (Morgens et al. 2017) The gRNA molecules I designed were 17 or 18 nucleotides long in order to take advantage of this increased specificity.

The method by which the gRNA and Cas9 are delivered greatly affects the ease and efficiency of CRISPR/Cas9 mutagenesis. Injecting the gRNA and Cas9 plasmid requires the least amount of preliminary work, but the method tends to have the lowest efficiency of all delivery methods (reviewed in Bassett and Liu 2014). Injecting a gRNA plasmid and Cas9 together results in a similar rate of mutagenesis to injecting gRNA oligos and Cas9 but is preferable because plasmids are more stable and easier to work with than RNA (Gokscezade et al. 2014). This is the method I decided to use in *D. simulans* as there are no pre-existing stocks of that species with the tools required to make pCFD4 integration by Φ C31. The method with the highest efficiency involves crossing a stock that produces gRNA(s) with another transgenic stock that produces Cas9. This method has been observed to cause mutagenesis in all progeny of the cross (Port et al. 2014), but producing the gRNA stock can often take months. I elected to use this method to induce a deletion in *Kat60* due to the high efficiency and the reduction in screening required after injection. The ability to produce a stable gRNA line was also attractive as it would allow future member of my lab to screen for *Kat60* deletions easily without having to microinject in case I was not able to finish this aspect of my project.

The two gRNA molecules I used targeted the 5' UTR and exon 2 of *Kat60* in the hope that they would excise the intervening region between both gRNA target sites. I was able to produce *D. melanogaster* flies that contained the full deletion between the two gRNA binding sites with an efficiency similar to those observed in other experiments that employed similar methods (Kondo and Ueda 2013; Ren et al. 2013; reviewed in Bassett and Liu 2014; Gratz et al. 2015). The main advantage of creating a large multi-exon deletion is that it ensures no functional *Kat60* protein could be produced from that allele. However, a similar effect could be achieved by using a single gRNA that targets the catalytic domain. Inducing an indel using NHEJ at this location could

prevent any functional protein product from being formed, similar to the *Kat60*^{17A} allele produced by Mao et al. (2014). This method would also be more efficacious as it only requires a single Cas9-induced DSB rather than two. This makes using a single gRNA especially attractive for *D. simulans* injections as the method used to induce mutagenesis in that species is less effective than the method used for *D. melanogaster*. However, the use of a single gRNA would require large amounts of screening and a complete lack of functional protein from that allele would be difficult to prove. In addition, there is also a chance that a mutation in the catalytic domain would not significantly affect function of the protein (if the mutation does not cause a frame shift, for example). Despite these drawbacks, this method might be worth pursuing if it remains difficult to produce *D. simulans* flies that contain the full deletion.

4.3 Testing *Kat60* RNAi Using Mating Assays

The mating assay protocol requires further optimization to determine the effects of silencing either the *mel* or *sim* allele of *Kat60* in certain tissues. While matings between *mel* females and *sim* males are easy to produce, it is extremely difficult to obtain hybrid offspring from these crosses when they contain a shRNA for *Kat60*. Larvae and pupae appear to develop normally, but hybrid offspring rarely progress past pupation. This resembles the phenotype produced by flies that lack a functional copy of *Kat60* (Mao et al. 2014), so it is possible that the shRNA is being driven too strongly at 28 °C. If this is correct, however, then the observed lethality would imply that the two shRNA constructs used to troubleshoot the assays (*mel*-exon5 and *sim*-exon6 because they were produced first) may not be allele-specific. Because both alleles of *Kat60* are similar, off-target binding of the guide strand to mRNA from the wrong allele could occur. High expression levels of shRNA can exacerbate off-target effects (Caffrey et al. 2011), so that rearing flies at 28 °C could drive expression to the point where the proportion of guide strands binding to the wrong allele is high enough to cause lethality. I am currently testing the viability of transgenic *mel/sim* hybrids reared at 24 °C and 20 °C to see if lowering the temperature can rescue the flies, but the assay should be run at the highest possible temperature to maximize efficacy of shRNA silencing.

4.4 Limitations

Despite my success producing transgenic lines, my work has several limitations. One of the most severe limitations is the time required to become proficient at microinjections and to produce stocks. As both aspects of my project (CRISPR and RNAi) relied on the ability to microinject embryos efficiently, a considerable amount of time was required to become skilled enough at microinjected to integrate plasmids or induce CRISPR mutations successfully. This was exacerbated by the fact that the original microinjection protocol required considerable changes to enhance optimization. Although I managed to optimize the protocol and integrate all my plasmids, a further 8–10 weeks was required to produce and expand the shRNA+Gal4 stocks for testing. I was only able to produce my first transgenic stock over a year after the start of my project, which greatly reduced the time available to gather data and troubleshoot. While a specialized company could have been used to perform these steps and decrease the time required to produce transgenics, the method is prohibitively expensive when injecting multiple constructs and complicated when the injections are desired in a species other than *D. melanogaster*.

The nature of the *mel* and *sim* alleles of *Kat60* also limited my ability to design shRNA. Algorithms have been developed to create potent shRNA constructs with limited off-target effects (Vert et al. 2006; Knott et al. 2014), but I was unable to utilize these tools because the shRNA sequences designed by these algorithms were not allele-specific. Because of this, the shRNA I designed are likely not as potent as shRNA produced by these algorithms.

While both pVALIUM20 and pCFD4 are excellent tools for RNAi and CRISPR respectively, both constructs have limitations. pVALIUM20 can induce strong silencing of target mRNA but the expression of shRNA can be leaky, especially when paired with a Gal4. The high expression of shRNA even at very low rearing temperatures (18 °C) can make it difficult to create or maintain some shRNA+Gal4 lines that target essential genes as the leaky expression can affect viability (Ni et al. 2011). The conventional method of using pCFD4 requires genome integration and crossing to a transgenic *mel* stock that produces Cas9. This method is an efficient way of producing gene knockouts, but it does not work in *sim* because of the relative lack of tools available in that organism. The construct must instead be injected into *sim* embryos along with a

Cas9 which has a far lower rate of mutagenesis (reviewed in Bassett and Liu 2014). The decision to use two gRNAs to create a large deletion in *Kat60* rather than using one and relying on NHEJ also reduced mutation efficiency, although such methods are likely necessary to be certain that the gene is nonfunctional.

4.5 Future Work

Substantial progress has been made on both aspects of my project, but much work remains. Several shRNA stocks still need to be produced and rearing conditions for sickly stocks need to be adjusted so that they can be expanded for use in mating assays. More pressing, however, is the need to characterize and create the *D. melanogaster* CRISPR deletion stocks as they are not yet stable and the deletion can be lost without careful screening. Even after a stable *D. melanogaster* stock is established, a *D. simulans* deletion stock is required before the reciprocal hemizyosity test can be performed to test the effect of *Kat60* on female receptivity (Stern 2014). This stock will be much more difficult to produce than the *D. melanogaster* deletion stock using my current method. A transgenic *D. simulans* stock containing an *AttP* site could be utilized in future methods (Stern et al. 2017b), but the lack of balancer chromosomes in *D. simulans* might prove troublesome, particularly as *Kat60* deletions are not homozygous viable.

Despite producing many transgenic stocks, I was unable to gather any mating assay data. The interspecies crosses caused unacceptable levels of hybrid lethality which could be due to leaky shRNA expression and/or nonspecific shRNA binding (Caffrey et al. 2011; Ni et al. 2011). This protocol must be improved before data determining the tissue-specific effects of *Kat60* can be gathered. The assay temperature should be lowered from 28 °C to reduce transgene expression and removing some of the UAS motifs present in pVALIUM20 should be considered if shRNA levels remain high even at low rearing temperatures. All shRNA stocks that I produced must also be tested for functionality through RT-PCR as it is uncertain whether they function as expected.

After these problems are resolved, I predict that the hybrids containing *mel*-targeting shRNA constructs, and thus predominantly producing *sim Kat60*, will be less receptive to mating attempts from *mel* males. The effect is likely to be strongest in hybrids that contain a *mel*--

targeting construct and *Act5C*- or *elav-Gal4* as these would silence the *mel* copy of *Kat60* in all neural tissues, though I would still expect to see a reduction in female receptivity when the shRNA is paired with *rut-Gal4* because of the mushroom body's effect on sensory integration and courtship memory (Montague and Baker 2016; Yagi et al. 2016). The contribution of each lobe of the mushroom body to female receptivity could be determined by using lobe-specific Gal4's. The *rut-Gal4* likely will not reduce female receptivity as strongly as *Act5C*- or *elav-Gal4* because other neural regions, such as the antennal lobe or the subesophageal ganglion, likely influence this trait as well (Rezával et al. 2012a; Sakurai et al. 2013). These regions should be tested to determine if they also mediate female receptivity through *Kat60*. If *Kat60* is shown to influence female receptivity in hybrids, it will be the first time a gene has been linked to heterospecific mate rejection, greatly increasing our understanding of the genetic basis of behavioural isolation. It will also indicate which neural regions influence female receptivity to heterospecifics and show how behavioural barriers to mating can develop between closely-related species.

4.6 Conclusions

I have produced multiple transgenic lines to determine the effect of *Kat60* on female receptivity towards heterospecific males. The protocol I generated to produce them is simple and can be learned to competency after a few months of practice. I designed four shRNA and one gRNA construct and integrated each into *D. melanogaster*. I have also produced stable stocks of each transgene. Three shRNA+Gal4 stocks have been produced for each shRNA to identify neural regions that influence female receptivity. Mating assay data could not be gathered due to severe hybrid lethality, but the shRNA stocks can be used immediately after those problems are resolved. I have not yet produced a *Kat60* deletion stock in *D. simulans*, which must be accomplished before the reciprocal hemizyosity test can be carried out. The stocks I have produced can be used to study the genetic basis of behavioural isolation between *D. melanogaster* and *D. simulans*, and the protocol I have developed can be used to study the function of nearly all genes in *D. melanogaster*.

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Appendix: Moehring Lab Microinjection Protocol

Making Apple/Grape Juice Plates

You will use apple or grape juice plates to collect fly embryos. Both types of juice work equally well. The recipe is as follows:

Ingredient	1L	2L	4L
Water	500 ml	1L	2L
Juice	500 ml	1L	2L
Agar	25g	50g	100g
Sugar	60g	120g	240g
Propionic Acid	3ml	6ml	12ml

Pour water, juice, and agar into the pot, begin heating the mixture, and stir constantly. This prevents the agar and juice from burning. Add the sugar and stir until all the granules are dissolved. Heat to a boil and wait for the solution to become clear, then take the mixture off the heat, add the propionic acid, stir, and pour into petri dishes. One litre of media usually makes 25–40 plates.

Setting Up the Fly Cage

Expand the stock you wish to inject until you have around 12–20 vials. Anaesthetize the flies with CO₂ and put them in one of the plastic fly cages. Take a juice plate and spread some yeast paste (active yeast granules mixed with water until smooth and the consistency of peanut butter) on it, then close the fly cage with it and seal with the orange cap. Put these flies in a 25°C incubator and leave them for a day to adjust to the cage. Changing the plates once or twice later in the day can help them adjust as well.

Pulling Loading and Injection Needles

Two types of needles are needed to inject: Injection needles, which are used to inject the embryos with DNA, and loading needles, which are used to load the injection mix into the injection needle. Loading needles are pulled by hand over a Bunsen burner. Borosilicate

capillary tubes (usually 1.0mm in diameter) are placed in the centre of the flame and rotated slowly. Once the glass becomes hot enough that the tube bends easily, quickly pull both ends apart to create a long, thin section in the middle. Take off the heat for about two seconds to allow the glass to harden and then break it in the centre with a razor. This will produce two loading needles. The point of these tubes should be able to reach the bottom of an injection needle, so it should be thinner than 0.75mm and at least 1.5 inches long. Secure them to a large petri dish with tape, label the dish, and bring the needles to the Biotron.

Injection needles are produced by using the needle puller in Dr. Greg Gloor's lab (MBL 8, send him an e-mail to ask if you can use it). Bring the 0.75mm borosilicate tubes from the Biotron to the puller to make the injection needles. Turn the needle puller on and select program 1 by pressing the "1" key. Then load your tube into the puller, align the centre on the heating filament, and secure the it at both ends with the thumbscrews. Close the UV shield and press the "pull" button. The puller will create two injection needles for every borosilicate tube that is pulled. Secure them to a large petri dish with tape (take care to not accidentally break the tip of the needle), label the dish, and bring it to the Biotron.

Preparing to Inject

Aliquot several microliters of your injection cocktail into a PCR tube and mix it with a microliter or two of food dye. This will allow you to easily visualize your injection mix entering the embryo. Then change the juice plate in the fly cage and set a timer for 30 minutes. During this time, load your injection needles and insert them into the microinjector. Turn the microinjector on and set the machine to "continuous" instead of "pulse". Change the injection pressure so that it is around 600 hPa (better to start with a low pressure and increase it than start with a high pressure, as this will kill your embryos), and set the transfer pressure to +50. This prevents the cytoplasm of the embryo from backflowing into the needle, which reduces survivability. Also, align the needle so that it is centered under the microscope. Prepare at least two needles to reduce the delay caused by a clogged or broken injection needle (which happens often).

Next, find or create an egg collection basket. These are made by taking a 50ml falcon tube, cutting it in half (keep the side with the cap), and then cutting a small square out of the cap. Take two pieces of 1.5" blue sheer ribbon, place them on top of the threaded opening, and screw

them into place using the cap. Ensure that the ribbon is pulled tight around the hole in the cap. The ribbon will act as a filter to catch embryos while allowing waste water to flow through. Finally, put a food plate (a petri dish filled with normal fly food) into an incubator to pre-warm it.

Injection Protocol

Once the 30 minutes are up, change the juice plate once more and keep the old one. You only have 30 minutes to inject a round of embryos so speed and efficiency are key. Rinse the old plate with distilled water to dislodge the embryos from the agar and pour them into the collection basket. Repeat once more to ensure that all embryos are collected. Wash the sides of the collection basket with water to collect any embryos that might have stuck to the side. Dry the ribbons with paper towel, remove it from the collection basket, and place under a microscope. You should see several embryos present. Take a coverslip and put a drop of distilled water on it. Using a damp paintbrush, align the embryos in the centre of the coverslip with the posterior end (the side without the two respiratory filaments) facing left. Try and become skilled enough to align at least 50 in 10 minutes. After alignment, remove most of the water from the embryos with a dry paintbrush or paper towel and move to the Biotron.

Cover the dry embryos with extra virgin olive oil (found in a 50ml falcon tube in the biotron or side room in the fly cave) and stick the coverslip to a microscope slide with a drop of water. Align the embryos vertically with their posterior end facing the needle. Slowly move the needle into the same vertical plane as the embryos, and gently brush the tip of needle on the chorion (egg shell) to open it (hold down the pedal so that you can see when the injection mixture first escapes the needle). Try to ensure that the break is as small as possible by moving the needle slowly and carefully. This causes the least amount of damage to the embryos and prevents backflow. The width of the needle tip should be less than 10% the width of the posterior end—I recommend using a different needle if this is not the case.

Inject the embryo by moving the stage toward the needle until the needle punctures the chorion. Ensure that the needle penetrated the egg and did not stop between the vitelline membrane (membrane surrounding the egg) and the chorion. Inject the embryo by pressing the pedal down or by holding “clear” and stop as soon as you see any food dye. Holding the “clear” button can cause the embryo to explode, so be extremely careful when using that method. If no

fluid escaped the needle, increase the pressure or hold “clear” to remove clogging. If the needle is still clogged, try to break it again on a chorion. If this does not help or is taking too long, use a new injection needle. After injection, quickly pull the needle out of the embryo. A good injection has little to no cytoplasmic leakage after removing the needle. Heavy leakage is a sign of a damaging injection and usually means that the embryo will not survive. Move the stage so that the needle is aligned with the next embryo and inject the next embryo in the same way.

Post-injection Care

After injection, tilt the coverslip vertically and use Kimwipes to drain as much oil as possible. Bring the coverslip back to the fly cave and wash the embryos with distilled water to remove the rest of the oil. Take care not to wash too vigorously as this can wash the embryos off the slide and ruin all your work. Dry the coverslip and embryos with Kimwipes as best as you can and stick them in the pre-warmed food plate so that the anterior end of the embryos is in direct contact with the food. Be careful not to crush the embryos against the food. You can fit multiple coverslips on a single plate if you are doing multiple rounds of injections per day. Surviving larva should be visible on the food within 1–2 days. After they develop into pupae, transfer each pupa into its own food vial for screening/crosses. If the injection stock is Bloomington Stock # 25709 or any other *vermillion* mutant stock, transgenic flies can be identified by the rescue of dark red/brown wild-type eyes (Figure 1).



Figure 1: Identifying transgenic offspring

Transgenic offspring can be recognized by their wild type eyes. The male (top) does **not** contain the transgene and has bright orange eyes indicative of *vermillion* mutants. The female (bottom)

has the transgene and darker wild-type eyes indicating *vermillion* rescue. Both flies were collected ~6 hours after eclosion and were produced by the same screening cross.

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Presentations

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