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The fruitless gene influences female mate preference in Drosophila

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Abstract

Species can arise as a result of reproductive barriers that prevent gene flow between diverging populations that force them to remain isolated from one another. Behavioural isolation is one of the earliest acting reproductive barriers determined by the evolution of mating preferences that prevent inter-specific matings. Several traits have been identified that contribute to behavioural isolation, but the genetic basis of interspecific female preference is yet to be determined. I used genetic mapping techniques to identify and confirm that the *fruitless* gene is affecting species-specific female rejection of interspecies males, contributing to the behavioural isolation between *Drosophila melanogaster* and *D. simulans*. I also determined that this species-specific female preference is caused by a non-sex specifically spliced transcript of *fruitless*. Transgenic rescue of *fruitless* expression using the *GAL4/UAS* system identified *fruitless* protein isoforms with a specific 3' C-terminal end are likely involved in species-specific female preference. Additionally, I discovered that female rejection of interspecies males is not determined by an individual sensory modality such as male courtship song or female perception of auditory and olfactory signals, and is likely controlled by the integration of multiple modalities. Finally, I constructed transgenic RNA interference lines to silence expression of specific *D. melanogaster* or *D. simulans fruitless* transcripts. These RNAi lines can be used to knockdown *fruitless* expression at specific developmental stages and in specific tissues using the *GAL4/UAS* system, and thus can be a useful tool for characterizing the genetic and neural mechanisms that govern species-specific female rejection.

Summary

Species are maintained as distinct groups by reproductive barriers that prevent interbreeding. Behavioural Isolation is one such reproductive barrier that acts via the incompatibilities in mating signals and preferences between diverged population groups. Our current understanding of the genetic mechanisms for behavioural isolation is limited. My research identifies the *fruitless* gene in *Drosophila* as a candidate gene for behavioural isolation. Through a series of genetic and molecular biological experiments I established that the *fruitless* gene is influencing female preference for mates within its own species, which is the first reported behavioural role in females for this gene. I also identified potential molecular and genetic mechanisms by which this gene is affecting female mating behaviour and contributing to the behavioural isolation observed between different species of *Drosophila*.

Keywords

Speciation, Behavioural isolation, female preference, *Drosophila, fruitless.*

Co-Authorship Statement

I completed the work of this dissertation under the supervision of Dr. Amanda Moehring. I received help from an undergraduate honours thesis student (A. Dhillon) in the collection of RT-PCR data (Chapter 2). Several of the deficiency and *P*-element lines used for deficiency mapping in Chapter 2 were completed by honours thesis students (Katrina Bruch, Co-Author, Chapter 2) and a former graduate student (Dr. Ryan Calhoun, Co-Author, Chapter 2). Transgenic deletions of *fruitless* exons were created by Dr. Megan Neville (Co-Author, Chapter 2). Finally, I received help from another graduate student (Joshua Issacson) in the creation of transgenic *fruitless* RNAi lines.

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List of Abbreviations

Act-5C	Actin 5C
Bal	Balancer chromosome
BDM	Bateson–Dobzhansky–Muller
Bon	Bonus protein
bp	base pair
BTB domain	BR-C, ttk, and bab domain
C1-C5	common coding exons 1-5 of *fru*
cac	*cacophany*
CHC	cuticular hydrocarbon
CNS	central nervous system
CRISPR	clustered regularly interspaced short palindromic repeats
Df	deficiency
DNA	deoxyribonucleic acid
DSB	double strand break
dsf	*dissatisfaction*
dsx	*doublesex*
F_1	offspring from the crossing amongst the P generation
fru	*fruitless*
Fru^M	male-specific *fruitless* protein

fru^{F}	female specific *fruitless* transcript
G_0, G_1	generation 0, 1
GFP	green fluorescent protein
HDAC1	histone deacetylase 1
HDR	Homology Directed Repair
HP1a	heterochromatin protein 1a
MiMIC	*Minos mediated integration cassettes*
NHEJ	non-homologous end-joining
Nlg1	*Neuroligin 1*
ORF	Open Reading Frame
P1-P5	5' exons 1-5 of *fru*
P53	*Tumor protein 53*
PCR	polymerase chain reaction
per	*period*
RNA	ribonucleic acid
RE	restriction enzyme
retn	*retained*
Sb	*Stubble*
shRNA	short hairpin RNA
Sh	*Shaker*

SNP	single nucleotide polymorphism
spin	*spinster*
SPR	*Sex peptide receptor*
Sxl	*sex-lethal*
sev	*sevenless*
Tb	*Tubby*
TE	Transposable element
tra	*transformer*
tra2	*transformer-2*
UAS	Upstream Activating Sequence
w	*white*
y	*yellow*
ZF	zinc-finger

Chapter 1

1 General Introduction

1.1 Speciation and Biological Species Concept

Understanding reproductive isolating mechanisms and how they contribute to the formation of new species is an important goal in speciation research. However, there is a lack of consensus on what constitutes a species and how they are characterized. Therefore, prior to examining speciation and its underlying processes, it is necessary to explore how species are defined.

There are numerous definitions of species that are based on distinct characteristics between one group of organisms and another. The Morphological Species Concept (Cronquist, 1978) categorizes species based on unique morphological characteristics. The Ecological Species Concept (Van Valen, 1976) defines species as lineages that occupy distinct adaptive zones or "niches" that are unique to that lineage. In contrast, the Biological Species Concept (Mayr, 1942) characterizes species as groups of interbreeding populations that are reproductively isolated from other such groups (Dobzhansky, 1935; Mayr, 1942).

The Biological Species Concept established reproductive isolation as the basis for speciation and fueled research aimed at characterizing its underlying mechanisms. Reproductive isolating mechanisms manifest as extrinsic or intrinsic barriers that prevent or significantly reduce gene flow between new populations separated by said barriers (Mayr, 1942; McNiven et al., 2011). The level of gene flow between populations is usually related to the geographical context of speciation (Mayr, 1963). For example, with a complete absence of gene flow between populations separated by physical barriers, and a lack of physical barriers in sympatric species (Nosil, 2008). Both of these are extreme scenarios, and typically gene flow is present at intermediate levels due to partial or incomplete nature of physical barriers (i.e. parapatric speciation; (Nosil, 2008)). Determining the presence of gene flow during speciation is difficult because the level of

genetic differentiation between taxa can be affected by the time of population divergence; the strength of reproductive barriers to gene flow or a combination of both (Nosil, 2008).

Although many theoretical models predict the feasibility of gene flow during speciation (Gavrilets, 2004; Bolnick and Fitzpatrick, 2007), there has been a lack of empirical evidence demonstrating this claim (Nosil, 2008). Recent studies comparing genetic variation across closely related species show that continuous gene flow during speciation may be a common occurrence under certain conditions (Niemiller et al., 2008; Nosil, 2008; Martin et al., 2013). This demonstration highlights the importance of intrinsic reproductive barriers (barriers that do not depend on the species' ecology) that contribute to the speciation process in the face of gene flow. Investigating the role of intrinsic reproductive barriers and the mechanisms by which they contribute to species isolation is an important first step towards clarifying the process of speciation and identifying the genes involved in species isolation.

1.2 Mechanisms of reproductive isolation

Reproductive barriers to gene flow can act before (prezygotic isolation) or after (postzygotic isolation) zygote formation (Mayr, 1963). The genetic basis of postzygotic isolating mechanisms such as hybrid sterility and inviability collectively referred to as hybrid incompatibilities has been studied extensively (Sasa et al., 1998; Turelli and Orr, 2000; Presgraves, 2002; Moehring, 2011; Dickman and Moehring, 2013). Hybrid incompatibilities result in a reduction in fitness of first generation (F_1) inter-specific hybrids and evolves through the accumulation of genes that cause incompatible interactions between the species (Presgraves, 2003; Maheshwari and Barbash, 2011). These incompatibilities are particularly advantageous in organisms with greater parental investment, or in cases where the energetic costs of bearing hybrids might reduce the likelihood of subsequent reproduction (Coyne, 1974). One of the first hybrid inviability genes identified was the X-linked oncogene *Xmrk* (Wittbrodt et al., 1989). In interspecific hybrid platyfish *Xiphophorus*, the gene interacts with an autosomal repressor gene, and results in the formation of lethal tumors (Orr et al., 2004; Scarpino et al., 2013). Phylogenetic analysis showed that a repeat region in the promoter of the repressor gene coevolved with the *Xmrk* oncogene simultaneously within these two *Xiphophorus*

lineages unlike standard BDM (Bateson–Dobzhansky–Muller) incompatibilities that are predicted to arise sequentially (Scarpino et al., 2013).

One of the most well characterized genes for hybrid inviability is the *Hybrid male rescue* (*Hmr*) gene in *Drosophila,* which results in the death of male hybrids from the cross between *Drosophila melanogaster* and its sibling species *D. simulans* (Barbash et al., 2000). It was later discovered that *Hmr* interacts with another gene, *Lethal hybrid rescue* (*Lhr*), to regulate heterochromatic modifications (i.e. telomere lengthening and overexpression of telomeric transposable elements) responsible for the differences in genome size and structure between *D. melanogaster* and *D. simulans* (Satyaki et al., 2014). Since hybrid lethality is suppressed by loss of function mutations in the *D. melanogaster Hmr* gene or in the *D. simulans Lhr* gene (Maheshwari and Barbash, 2011), it is likely that the lethality in observed in hybrids is a result of epistatic interactions unique to the hybrid background (Satyaki et al., 2014).

Hybrid male sterility typically evolves before hybrid female sterility or hybrid lethality in diverging species (Turissini et al., 2017; Bundus et al., 2018). In *Drosophila* species pairs there is greater incidence of hybrid male sterility than female sterility, which is most likely due to genetic conflicts and incompatibilities in hybrid spermatogenesis (Wu and Davis, 1993; Orr et al., 2004). Through a series of classical genetic mapping techniques, the *Odysseus site homeobox* (*OdsH*) gene was the first locus identified to cause hybrid male sterility, in F1 hybrids between *D. mauritiana* and *D. simulans*. Since then, a number of potential hybrid sterility loci have been identified in *Drosophila* and other model systems such as *Mus musculus* and *Caenorhabditis elegans* (Bundus et al., 2018; Mihola et al., 2009; Tao et al., 2003). For example, the *PRDM9* gene is shown to cause hybrid sterility between the subspecies *Mus musculus musculus* and *Mus m. domesticus* (Mihola et al., 2009).

When genetic divergence leads to partial post-zygotic isolation in allopatry, secondary contact might drive selection for traits that prevent maladaptive hybridizations. This can result in females discriminating against males from different species via pre-zygotic behavioural isolation to avoid the production of sterile or inviable hybrids, or hybrids that

are otherwise maladapted (Dobzhansky, 1940; Schluter, 1995). This can reinforce local adaptation and habitat-based isolation (Schluter, 1995).

Pre-zygotic isolation includes reproductive barriers that prevent successful mating or fertilization and evolves earlier than post-zygotic barriers in both plants (Grant, 1992; Ramsey et al., 2003) and animals (McMillan et al., 1997; Sánchez-Guillén et al., 2014). Pre-zygotic barriers also undergo stronger selective pressure in the presence of post-zygotic barriers (Orr and Coyne, 1989; Liou and Price, 1994; Coyne and Orr, 2004). The pre-zygotic barrier of habitat isolation is a direct result of a preference for distinct habitats, reducing or preventing interspecific encounters (Rice and Salt, 1990). Habitat isolation can manifest in niches separated by space and time, as in the case of herbivorous insect populations adapted to distinct host plant niches that vary spatially and temporally (reviewed in Funk et al., 2002). Species within the apple maggot fly *Rhagoletis pomonella* sibling complex are a model for sympatric speciation via host plant shifting (Feder et al., 2003). Different host-specific populations have an overlapping geographical distribution (i.e., are partially sympatric) but remain reproductively isolated by occupying different host plants (Feder et al., 2003). In addition, they are further isolated by maintaining differences in the timing of their pupal diapause (Linn et al., 2004; Hood et al., 2012). There is also evidence of sexual isolation among *Rhagoletis* species as a result of behavioural preferences for different fruit odors, as mediated by chemosensory cues (Linn et al., 2004). Although pre-zygotic post-mating isolation (i.e. conspecific sperm preference) likely evolved much later than geographical and ecological barriers in *Rhagoletis* species, there are cases where it evolves earlier in the divergence process (e.g. *Neochlamisus bebbianae*; leaf beetles). As such, pre-zygotic post-mating barriers can act as a strong initial barrier to gene flow and speciation (Funk et al., 2002; Rundle and Nosil, 2005; Hood et al., 2012).

Behavioural isolation is another form of pre-zygotic isolation that involves a lack of sexual attraction due to incompatibilities in sexual signals between diverged populations. Among pre-zygotic isolation mechanisms, behavioural isolation evolves at faster rates compared to post mating pre-zygotic barriers such as gametic isolation, (Ludlow and Magurran, 2006) and post-zygotic barriers like hybrid sterility and inviability (Coyne and

Orr, 1997, 2004; Turissini et al., 2017). In the absence of geographical, temporal or ecological barriers, divergence in mating signals and preferences between populations can result in behavioural isolation and contribute to the speciation process (Selz et al., 2014).

1.3 Behavioural isolation

Successful mating usually involves the exchange of sexual signals between males and females (Coyne and Orr, 2004). In most animal species, the male presents a signal that elicits a preference from females of their own species (the signal is intraspecific) but not females of another species. The male signal and corresponding preference from the female likely co-evolve (Rodríguez et al., 2006; Moehring and Boughman, 2019), leading to positive assortative mating for mutual preference (Alpern and Reyniers, 1999). The co-evolution of signals and their preference can ultimately result in species isolation (Ting et al., 2001; Coyne and Orr, 2004).

Courtship behaviour is a complex trait involving multimodal signaling (Griffith and Ejima, 2009). For instance, female pigeons, *Columba livia*, preferentially respond to a combination of visual and auditory signals from the male than from any individual component of male courtship (Partan et al., 2005). In species of *hylids* (frogs), *bufonids* (toads) and *microhylids* (narrow mouthed toads), females discriminate between intra- and interspecific males based on species-specific mating calls (Gerhardt, 1974). In African lake cichlids, colouration patterns and visual acuity contribute to female preference for intraspecific males, and discrimination against interspecific males (Seehausen and van Alphen, 1998; Couldridge and Alexander, 2002; Maan et al., 2004, 2006;). The sexually dimorphic fish species *Etheostoma barrenense* and *E. zonale* is another instance where females showed a preference for conspecific over heterospecific male nuptial colouration and patterning, which may contribute to behavioural isolation between these species (Williams and Mendelson, 2011).

Chemosensory signals also play an important role in courtship in many insect species. For instance, within the two reproductively isolated species *Chorthippus biguttulus* and *C. mollis,* surface compounds called cuticular hydrocarbons (CHCs) from females induce

acoustic courtship signals from conspecific but not heterospecific males. This pattern suggests that the males assess species-specific olfactory cues from females and respond with species-specific courtship song. The males' assessment of female cues and their subsequent response indicates that courtship in these species involves multimodal signals and that signaling play a role in the behavioural isolation of these species (Finck et al., 2016).

Within *Drosophila* species, signals that determine mate choice can contribute to mating isolation between species pairs. *Drosophila* females use visual cues to assess male courtship, and certain visual cues can affect female receptivity (Cook, 1979). For example, females of the Hawaiian fruit fly species *Drosophila heteroneura* are less receptive to *D. silvestris* males, which lack the elaborate physical ornamentation of *D. heteroneura* males (Boake et al., 1997). Similarly, auditory or olfactory signals like the "wing-song" of *Drosophila* males or specific CHCs, respectively, possess specific characteristics that affect species-specific recognition and thus may contribute to behavioural isolation (Tomaru et al., 2000; Billeter et al., 2009; Bontonou et al., 2012). There are numerous studies that have linked specific mating cues to behavioural isolation across multiple taxa, and empirical studies exploring the genetic basis of behavioural isolation (Reviewed in Ptacek, 2000; Rosenthal, 2013).

1.4 Genetic basis of behavioural isolation

A number of genes are likely involved in shaping the intricate exchange of the co-evolved signals and preferences between the sexes (Andersson and Simmons, 2006; Endler and Houde, 1995; Kirkpatrick, 1982; Miller and Pitnick, 2002). Several traits involved in mating behaviour have been linked to species-specific mating success (Seehausen and van Alphen, 1998; Haesler and Seehausen, 2005; Rafferty and Boughman, 2006; Nosil and Schluter, 2011; Conte and Schluter, 2013). One of the earliest examples of a definitive link between mate preference and genetic variation was observed in the two-spot ladybird species *Adalia bipunctata*. In this case, females from diverged populations showed a significant preference for melanic male patterns, which were attributed to a single dominant locus (Majerus et al., 1982).

In *Heliconius* butterfly species *H. cydno* and *H. pachinus*, assortative mating is influenced by the divergence of male preference for female wing colour patterning (Kronforst et al., 2006). Quantitative trait locus mapping revealed that a preference locus is linked with the locus that determines forewing colour. Suggesting a potential gene influencing female preference for forewing colour (Kronforst et al., 2006). Additionally, yellow-coloured males from the polymorphic race *H. cydno alithea* prefer to court females of the same colour, indicating that colour pattern and colour preference might be under the control of a single pleiotropic locus or multiple loci located within a chromosomal inversion (Kronforst et al., 2006; Gray and McKinnon, 2007). This type of close genetic association between a signal and its preference increases the likelihood of divergence and speciation, as compatible preference and trait alleles are more likely to be inherited together. This close genetic linkage of signal and preference loci suggests a coevolution of signals an preferences involved in species-specific mate recognition (Kronforst et al., 2006).

The Hawaiian cricket species *Laupala paranigra* and *L. kohalensis* have been the subject of extensive research into behavioural isolation. These two species vary in courtship song pulse intervals, and females have a unimodal preference for intraspecific pulse rates (Shaw, 1996, 2000). Assessing pulse rates and female preferences in hybrids and backcrosses revealed a genetic correlation between courtship song and female preference (Shaw and Lesnick, 2009). Additionally, two quantitative trait loci for song pulse variation overlapped the loci for female preferences (Wiley and Shaw, 2010). This suggests that there is widespread linkage between loci underlying male courtship signaling and female preferences for these signals in *Laupala* and is indicative of how intersexual communication can affect assortative mating and contribute to speciation (Wiley and Shaw, 2010)

Drosophila is a commonly used model for studying the genetic basis of behavioural isolation. There is strong evidence for functional genetic variation between *Drosophila* species (Zeng et al., 2000). The genetic mechanisms of mating behaviour have been thoroughly studied in the genus. This has led to the discovery of the genes *period* (Kyriacou and Hall, 1980), *doublesex* (Baker et al., 1989; Burtis and Baker, 1989), and

fruitless (Hall, 1978; Gailey and Hall, 1989), which affect different stages of male courtship. Similarly, the genes *dissatisfaction* (Finley et al., 1997), *chaste* (Juni and Yamamoto, 2009), and *spinster* (Suzuki et al., 1997) reduce female receptivity to courtship when they are mutated (Carracedo et al., 2000a; Laturney and Moehring, 2012a) . It remains unclear whether these genes play a role in species-specific mating behaviour. One study by Carracedo *et al.* (1989) proposed that both intra- and interspecific female preference might be controlled by the same genetic loci. However, more recent studies indicate that species-specific female preference might be determined by the interaction of several genes that are not involved in intra-specific female preference (Gleason and Ritchie, 2004; Gleason et al., 2009; Laturney and Moehring, 2012a).

In *Drosophila,* as with many species, copulation success ultimately depends on female receptivity and willingness to mate with males after evaluation of the male's courtship (Greenspan, 1995). A small number of loci have been identified as candidate genes for natural variation in female preference both within (Finley et al., 1997; Suzuki et al., 1997; Juni & Yamamoto, 2009; Laturney & Moehring, 2012a) and between species (Campesan et al., 2001; Ting et al., 2001; Fitzpatrick et al., 2005; Kronforst et al., 2006; Moehring et al., 2006; Laturney & Moehring, 2012). However, very few loci have been fully explored for their role on species-specific female preference and the mechanism by which these loci affect this trait.

1.5 *Drosophila* as a model organism

The fruit fly *Drosophila* has been a cornerstone of genetic research for over 100 years starting with the groundbreaking work of T.H. Morgan, whose research established the theory of chromosomal inheritance and genetic linkage (reviewed in Jennings, 2011). Although the genus *Drosophila* encompasses 1579 known species (Brake and Bächli, 2008), the vast majority of research is centered on the cosmopolitan species *Drosophila melanogaster*, which is a prominent model for the study of behaviour, development, neurobiology and human disease. Compared to most vertebrate models, *Drosophila* has technical advantages, including: short generation times; large cost-effective sample sizes; easy to culture in the laboratory and large number of externally-developing embryos

(Jennings, 2011). *Drosophila melanogaster* was one of the first models with a fully sequenced and well-annotated genome, which is publicly available in Adams et al. (2000). Over the years, the genomes of multiple strains of *D. melanogaster* as well as additional *Drosophila* species, have been sequenced and made available (Ashburner and Bergman, 2005). The multiple genome sequences were used to generate a detailed single nucleotide polymorphism (SNP) map, and contributed to the creation of genomic, EST, and cDNA libraries. These, in turn, facilitated a range of additional genetic tools available in *D. melanogaster*, including a variety of genetic elements that have been integrated into the genome.

However, traditional recombination mapping techniques cannot be used in *D. melanogaster* for behavioural isolation studies because this species does not produce fertile hybrids with any of its sibling species (Coyne, 1992a; Doi et al., 2001), preventing the production of recombinants. This drawback was recently circumvented by using deficiency mapping, which can be used to map recessive traits (Pasyukova et al., 2000; Mackay, 2001)

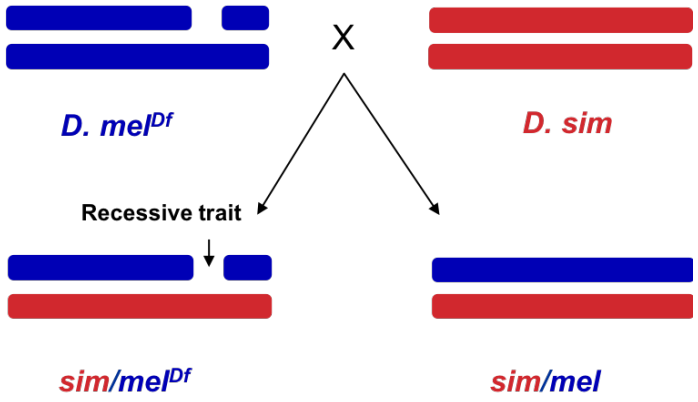

Figure 1-1 Schematic of Deficiency mapping (blue and red lines represent chromosomes). *D. melanogaster* (*mel*, top left) females bearing a deficiency (broken blue line) are crossed to *D. simulans* (*sim*, top right; red lines) produce hybrids inheriting intact chromosomes from both species (bottom right; *sim/me*) and hybrids inheriting a disruption in the *D. melanogaster* chromosome (bottom left; sim/mel^{Df}). Any *D. simulans* recessive traits in this region, will be unmasked in these hybrids with the deficiency.

Deficiency mapping utilizes chromosomal deletions to unmask recessive traits in F1 hybrids between species (Pasyukova et al., 2000; Figure 1-1). Subsequent smaller overlapping deletions can be used to map recessive traits down to a single locus. Deficiency mapping stocks have deleted regions (deficiencies) at defined chromosomal locations, which overlap partially with deficiencies in other stocks. Three major projects undertaken by the BDSC (Bloomington Drosophila Stock Center), Exelixis, Inc. and the DrosDel project have generated a library of deficiency mapping stocks that provide 98.4 % coverage of the *D. melanogaster* genome (Cook et al., 2012; Roote and Russell, 2012).

Another important resource in *D. melanogaster* is the large collection of individual gene disruptions available for the vast majority of genes in *D. melanogaster* (Spradling et al., 1999; Bellen et al., 2004a). These can be useful in reverse genetics screens to identify gene function by analyzing phenotypic effects of mutated genes. They also allow the testing of individual candidate genes for behavioural isolation by using the same approach as deficiency mapping, but now using a single gene mutation instead of a large chromosomal deletion (Laturney and Moehring, 2012a).

These gene disruptions were primarily created using transposable elements. These include mobile genetic elements inserted into the *D. melanogaster* genome, such as *P* (Spradling and Rubin, 1982), *Minos* (Franz and Savakis, 1991), and *piggyback* (Handler and Harrell Ii, 1999). The Berkeley *Drosophila* Genome Project (BDGP) gene disruption project generated over 30,000 fly strains, each with a transposable element inserted in a gene, located throughout the *Drosophila* genome (Bellen et al., 2004b). Currently more than 9,000 genes have been disrupted with various transposable elements, with many genes having multiple insertion sites within the locus (Bellen et al., 2011). This gene disruption library is particularly useful for reverse genetic screens, since the location of the insertion within a gene can have variable effects, and testing multiple disruptions within a candidate gene can reveal how the gene functions (Spradling et al., 1999; Bellen et al., 2004b).

The primary goal of transposon-mediated mutagenesis is to disrupt gene function, but these mutations can also be engineered to characterize the function and expression of

enhancers (enhancer traps) or disrupt genes and determine their expression patterns using gene traps and protein traps (Venken et al., 2011). Additionally, transposable elements can incorporate recognition sites for recombinase-mediated cassette exchange (RMCE) to replace sequences within the transposons with other sequences of interest (Schlake and Bode, 1994; Venken et al., 2011). For example, *Minos* mediated integration cassettes (*MiMIC*) are highly modular transposon insertions that function as gene traps and that have recognition sites for integrases. These recognition sites allow easy exchange of the gene trap cassette with any other sequences such as protein tags, reporter genes, and enhancer traps (Venken et al., 2011).

One of the most powerful tools for studying targeted gene expression in *Drosophila* is the *GAL4/UAS* system (Fischer et al., 1988). This is a versatile tool that can be used to express a gene of interest in a particular tissue or a specific stage of development. This system uses the yeast transcription factor *GAL4* to activate transcription of genes by binding to an upstream activating sequence (*UAS*). In *D. melanogaster*, the *GAL4* ORF is inserted into the genome, usually near a promoter or enhancer. This results in the expression of *GAL4* protein in a tissue specific pattern under the control of that promoter or enhancer. The *GAL4* protein binds to the *UAS* sequence and activates transcription of any gene or reporter downstream of the *UAS* sequence in a targeted tissue specific manner (Duffy, 2002). Pairing *GAL4* with a *UAS*-GFP, which expresses a green fluorescent protein, allows for the visualization of the expression pattern for nearby promoters and enhancers that affect the *GAL4*. If the *GAL4* is inserted next to the promoter of a gene of interest, this can allow for ready visualization of that gene's expression pattern. Pairing this *GAL4* with other types of *UAS* constructs, such as those that induce apoptosis or neural hyperactivation, provides the ability to manipulate the cells in which the gene of interest is expressed. Lastly, pairing a range of different *GAL4s* with a *UAS* linked to a target gene of interest, and observing the effect of the expression of this gene in specific tissues or at specific times, can reveal when, where, and how the gene functions.

In addition to the vast array of genetic and molecular tools available, the *Drosophila* genus has multiple closely-related species that are sexually isolated (Figure 1-2), but can

be mated within the laboratory (Jennings and Etges, 2010). Several species pairs exhibit varying levels of species-specific female preference (Blows and Allan, 1998; Coyne and Orr, 1997). Moreover, the evolutionary relatedness of these species to *D. melanogaster* means that the genome sequence and gene annotations from *D. melanogaster* can be informative when characterizing genes and functions within closely related species.

1.6 Courtship behaviour in *Drosophila*

Drosophila courtship is a complex innate behaviour that involves the exchange of multiple sensory inputs between the male and the female. *Drosophila melanogaster* males initiate courtship after they perceive a potential mate through visual, olfactory and gustatory inputs (Waldron, 1964; Ewing and Bennet-Clark, 1968; Spieth, 1974; Cowling and Burnet, 1981; Greenspan and Ferveur, 2000; Nguyen and Moehring, 2019). Males then display a fixed sequence of behaviours that include following the female, tapping her abdomen with his forelegs, vibrating his wings to produce a species-specific courtship "song", licking her genitalia with his proboscis and finally mounting the female and bending his abdomen to attempt copulation (Spieth, 1974; Villella and Hall, 2008; Pavlou and Goodwin, 2013). The female evaluates the cues during courtship and makes a decision to either copulate with males based on the quality and species-specific nature of cues or reject males by extruding her ovipositor, kicking and flicking her wings, or walking away (Connolly and Cook, 1973; Spieth, 1974; Cobb and Jallon, 1990).

Courtship behaviour can vary considerably among *Drosophila* species groups, often comprising of unique movements or species-specific signals (Spieth, 1974). For example, *D. virilis* males extend their wings 10-14° and vibrate them vertically at smaller amplitudes of displacement. In contrast, *D. melanogaster* males extend their wings 90° and vibrate them vertically at larger amplitudes. Finally, *D. planitibia* males extend their wings up to a 160° and simultaneously vibrate them horizontally and vertically (Spieth, 1974). As a result, the male courtship song can vary qualitatively and quantitatively between species (Ewing and Bennet-Clark, 1968). For example, courtship song in the *melanogaster* species group typically consists of trains of pulses ("pulse song") with inter-pulse intervals (IPI) and sinusoidal ("sine song") waves (Ewing and Bennet-Clark, 1968; Kyriacou and Hall, 1980). The intervals in *D. melanogaster* courtship songs are

shorter than those of *D. simulans* courtship songs, and females detect this acoustic component and use it to identify conspecific males (Ewing & Bennet-Clark, 1968; Von Schilcher, 1976a; Kyriacou & Hall, 1980). A detailed study by von Schilcher, 1976b showed that *D. melanogaster* females paired with wingless "muted" males will mate more quickly if recordings of the *D. melanogaster* species-specific pulse song are played, but not if the *D. simulans* pulse song is played. This behavioural preference suggests that the inter-pulse intervals of courtship song play an important role in mate recognition and assortative mating, resulting in mating isolation.

Drosophila CHCs primarily function for desiccation resistance but also function as pheromonal compounds that are involved in intra- and interspecies sexual communication (Jallon and David, 1987). The CHC composition can vary significantly among species and different strains within the same species. For example, the pheromone 7,11-heptacosadiene is the predominant compound present on *D. melanogaster* females only, but is not present on males. In contrast, 7-tricosene is primarily present in *D. melanogaster* males and both *D. simulans* sexes (Veltsos et al., 2012; Pardy et al., 2019). Within *D. simulans*, strains from West Africa express 7-pentacosene as the most abundant CHC compound as opposed to 7-tricosene that is common in *D. simulans* strains in North America (Bontonou et al., 2012). The sexually dimorphic nature of CHCs and the variation between species indicates that CHCs are involved in both sex and species identification. For example, *D. melanogaster* males court *D. melanogaster* males that lack oenocytes (cells that produce CHCs) with the same intensity as they court *D. melanogaster* females. However, when these oenocyte-less males were coated with male-specific *D. melanogaster* pheromone (7-tricosene), the males no longer courted them (Billeter et al., 2009). Similarly, oenocyte-less *D. melanogaster* females are courted at high levels by *D. simulans* males until these females are coated with the *D. melanogaster* female CHC, 7-11-heptacosadiene (Billeter et al., 2009).

Fabre et al. (2012) recently discovered a new component of *D. melanogaster* courtship. During courtship, the male abdomen vibrates, and the vibrations are transmitted to the substrate surface via the legs at about six pulses per second. Females sense these vibrations and stop walking, and allow the male to attempt copulation (Fabre et al.,

2012). The use of substrate vibrations to court females is conserved in several *Drosophila* species, some from the *D. melanogaster* subgroup (*D. biarmipes*, *D. mauritiana*, and *D. simulans*), and others from more distant groups (*D. mojavensis* and *D. willistoni*) (Fabre et al., 2012; Mazzoni et al., 2013). Substrate-borne vibrations are species-specific, and it is possible that this particular courtship signal might have a role in species-specific female preference.

1.7 Behavioural isolation in *Drosophila*

Some degree of behavioural isolation is observed between most *Drosophila* species pairs (reviewed in Nanda and Singh, 2012). This isolation can be complete, with no matings occurring between species, or incomplete, where some matings occur. For example, the Hawaiian species *D. silvestris* and *D. heteronuera* are sympatric but exhibit incomplete behavioural isolation due to failure of male *D. silvestris* to initiate courtship with female *D. heteroneura* (Boake et al., 2000). Behavioural isolation between these two species occurs at the initial stage of courtship when the male first approaches the female, and it is the critical stage of species recognition in this species pair. This observation suggests that in this species pair, behavioural isolation is attributable to males' decision to initiate or avoid courtship (Price and Boake, 1995; Boake et al., 1997, 2000).

Behavioural isolation is commonly observed between species living in sympatry (Orr and Coyne, 1989). The North American species *D. pseudoobscura* and *D. persimilis* are sympatric and behaviourally isolated. However, *D. pseudoobscura* populations that live allopatrically to *D. persimilis* do not exhibit strong behavioural isolation (Noor, 1995). This suggests that, in the sympatric populations, *D. pseudoobscura* females evolved an increased sexual isolation as a result of maladaptive matings with interspecific males. QTL mapping identified two loci, *Coy-1 and Coy-2*, associated with *D. pseudoobscura* female discrimination of *D. persimilis* males (Ortiz-Barrientos et al., 2004). When the sympatric allele for one of the identified loci (*Coy-2*) was introgressed into *D. persimillis* from an allopatric population, there was increased discrimination from these allopatric *D. persimillis* females towards *D. pseudoobscura* males (Ortiz-Barrientos et al., 2004). This likely corresponds with a one-allele model where the presence of just one allele, can increase discrimination between species (Ortiz-Barrientos et al., 2004).

The largest body of work on behavioural isolation in *Drosophila* has been done with the *D. melanogaster* subgroup and the *D. simulans* complex (Figure 1-2). The recently diverged species of the *simulans* complex (*D. mauritiana* and *D. sechellia*) have been studied comprehensively owing to the complex network of asymmetric mating preferences. The ability to produce fertile hybrids allows for recombination mapping to identify traits and genomic regions involved in species-specific female preference (Coyne et al., 1994; Coyne and Orr, 2004; Moehring et al., 2004; McNiven and Moehring, 2013; Le Vasseur-Viens et al., 2015). However, none of the species within this group have the genetic tools available in *D. melanogaster*. The recently utilized approach of deficiency mapping within F1 hybrids of *D. melanogaster* and *D. simulans* has made this species pair available as a model for identifying the genetic underpinnings of behavioural isolation and species-specific female preference.

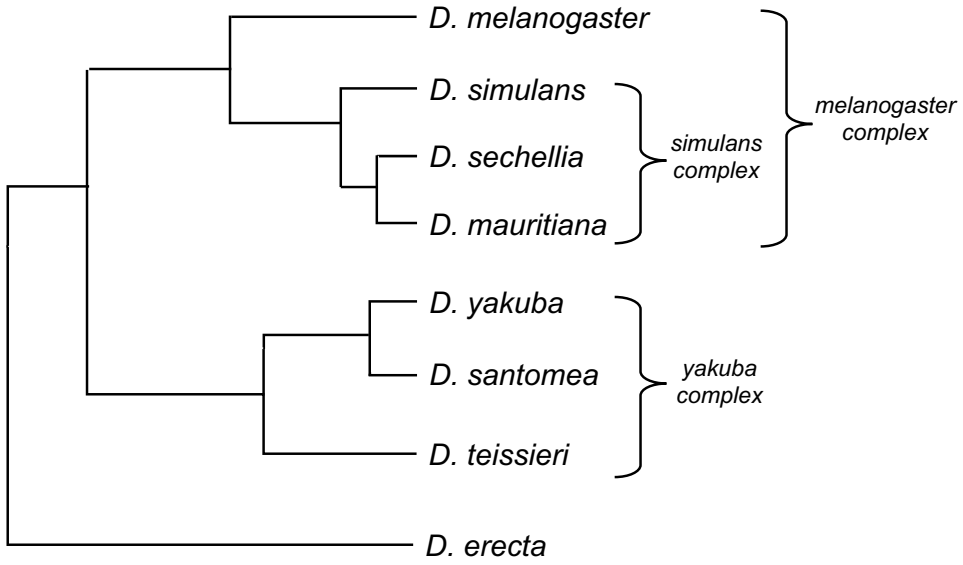

Figure 1-2 Phylogenetic tree of the melanogaster subgroup species. The *melanogaster, simulans* and *yakuba* species complexes are depicted. Figure adapted from (Ambrosi et al., 2013).

Drosophila melanogaster and *D. simulans* are sister species from central Africa that diverged approximately 5.4 million years ago (Tamura et al., 2004). Over time, these

species have migrated with human populations to achieve a global distribution. These two species are asymmetrically isolated from one another, where mating between female *D. simulans* with male *D. melanogaster* rarely occurs (Sturtevant, 1920; Barker, 1967; Carracedo et al., 2000b) but *D. melanogaster* females mate at moderate frequency with *D. simulans* males if placed in a no-choice assay, producing inviable hybrid males and sterile hybrid females (Sturtevant, 1920; Manning, 1959). Since the hybrid females are mildly receptive towards *D. melanogaster* males (Davis et al., 1996), loci contributing to *D. melanogaster* receptivity are likely semi-dominant over the corresponding *D. simulans* non-receptive loci. Previous studies within the *melanogaster* subgroup that utilized chromosomal substitutions and QTL maps found genomic regions involving species-specific female preference localized on all three chromosomes. However the right arm of the third chromosome (3R) was consistently linked to species-specific preference in multiple species pairs (Coyne, 1992; Carracedo, Pineiro, & Casares, 1995; Uenoyama & Inoue, 1995; Ting et al., 2001; Moehring et al., 2004) in multiple species pairs. Genetic analysis of behavioural traits such as courtship song showed that ~41% of genes involved in variation in male courtship song are also localized on 3R (Gleason, 2005). Similarly, four different loci contributing to pheromonal differences between *D. melanogaster* and *D. simulans* were mapped to the right arm of the third chromosome (Coyne, 1996).

Genes contributing to behavioural isolation are likely to localize in regions of low recombination, near centromeres, telomeres and interspecific inversion polymorphisms (Noor et al., 2001; Nanda and Singh, 2011). Regions of low recombination between interbreeding populations allow for the maintenance of population-specific gene complexes. Accumulation of new mutations within these complexes, in addition to the reduced rate of recombination in these areas, can result in a population-typical phenotype if the complexes contain variants for local adaptation (Stevison et al., 2011; Laturney and Moehring, 2012b)

The *D. melanogaster* and *D. simulans* genomes differ by approximately 3% and a large inversion polymorphism (Ranz et al., 2007). This inversion polymorphism is also on 3R, the same chromosome arm implicated through genetic mapping as strongly contributing to behavioural isolation. Fine scale deficiency mapping of 3R identified five genomic

regions that might harbour loci involved in species-specific female preference (Laturney and Moehring, 2012a). Former students from the Moehring lab performed additional fine-scale deficiency mapping within these regions, and tested individual genes to identify candidate genes for both female preference and behavioural isolation. One of the candidate loci that was identified through fine-scale genetic mapping for behavioural isolation was the gene *fruitless*.

1.8 The *fruitless* gene

The *fruitless* gene *(fru)* is an important regulator in the sex determination hierarchy and is highly conserved across the orders Diptera (Davis et al., 2000), Hymenoptera (Bertossa et al., 2009), and Blattaria (Clynen et al., 2011). This suggests an ancient origin and a common function among different insects. The sex determination hierarchy genes, *Sex-lethal (Sxl), transformer (tra),* and *transformer-2 (tra-2)* regulate somatic sexual differentiation (Burtis, 1993). In *Drosophila* sex is determined by the ratio of autosomes to X chromosomes (Figure 1-3; Nöthiger and Steinmann-Zwicky, 1985). In females this ratio (X:A=1) determines whether *Sxl* is transcribed and produces protein, which subsequently regulates the downstream splicing of *tra* and the production of functional Tra protein. Tra and Tra-2 then regulate the downstream female-specific splicing of *doublesex* (*dsx*) and *fru*. In males, *Sxl* is not produced, and therefore *dsx* and *fru* pre-mRNAs undergo the default male-specific splicing. *Doublesex* proteins act as sexually dimorphic transcription factors that regulate external morphological features and internal biochemistry (Ryner et al., 1996), while the male *fruitless* proteins regulate different aspects of male sexual differentiation and behaviour (Greenspan, 1995; Ryner et al., 1996).

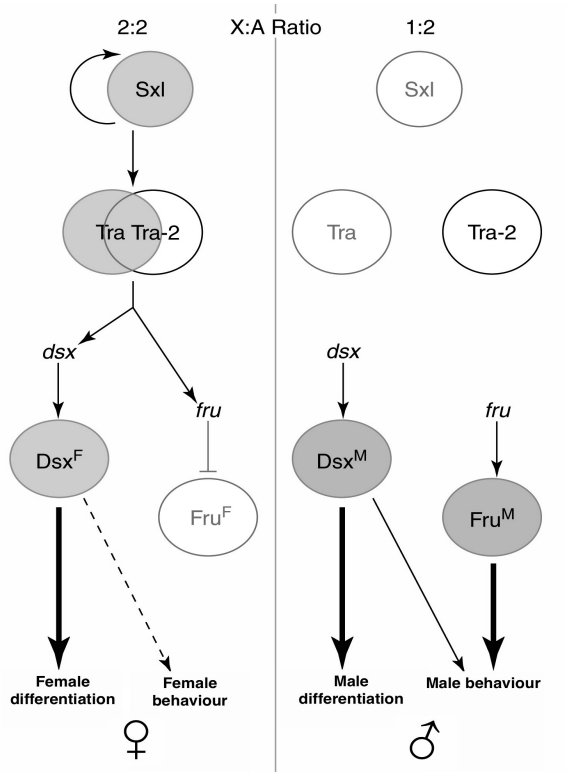

Figure 1-3 The sex determination hierarchy in *Drosophila*. In females, the *Sex lethal* gene is activated by auto-regulatory splicing of its own pre-mRNA. Sex Lethal (Sxl) protein initiates female-specific splicing of *transformer* to generate the Transformer (Tra) protein. Tra, and Transformer-2, (Tra-2), regulates female-specific splicing of *doublesex* (*dsx*) and *fruitless* (*fru*). The dsx^F mRNA is translated into Dsx^F protein but the female-specific fru^F mRNAs are not translated (Usui-Aoki et al., 2000). Dsx^F regulates female somatic differentiation, external morphology, and female-specific behaviours (Baker et al., 2001). In males, absence of Tra causes *dsx* and *fru* to be spliced into functional male specific Dsx^M and Fru^M proteins. Dsx^M controls the formation of male specific structures and external morphology (Burtis and Baker, 1989). Dsx^M has also been shown to influence the development of specific Fru^M-expressing neurons in the male nervous system (Billeter et al., 2006a). The Fru^M protein is required for the development of male sexual behaviour (Billeter et al., 2006a; Demir and Dickson, 2005; Ryner et al., 1996). Figure adapted from Billeter et al. (2006).

Disruptions in *fru* affect multiple aspects of male courtship behaviour. *Drosophila melanogaster* males with a mutation in *fru* that causes female-specific splicing will court one another to form courtship chains (Hall, 1978). Males that have a complete deletion of the sex-specific transcript of *fru* do court females, but rarely attempt to copulate, and are never successful when they do, thus they are considered to be behaviorally sterile (Hall, 1978; Ito et al., 1996; Usui-Aoki et al., 2000). Males with sex-specific *fru* mutations also stimulate courtship from wild-type *D. melanogaster* males (Hall, 1978). However, the importance of *fruitless* extends beyond male courtship and sex determination, as it is shown to also be necessary for external morphology such as the formation of the male specific "Muscle of Lawrence" (MOL) (Gailey et al., 1991; Lawrence and Johnston, 1984), which is required for the male's ability to curl its abdomen during copulation attempts (Gailey et al., 1991; Usui-Aoki et al., 2000).

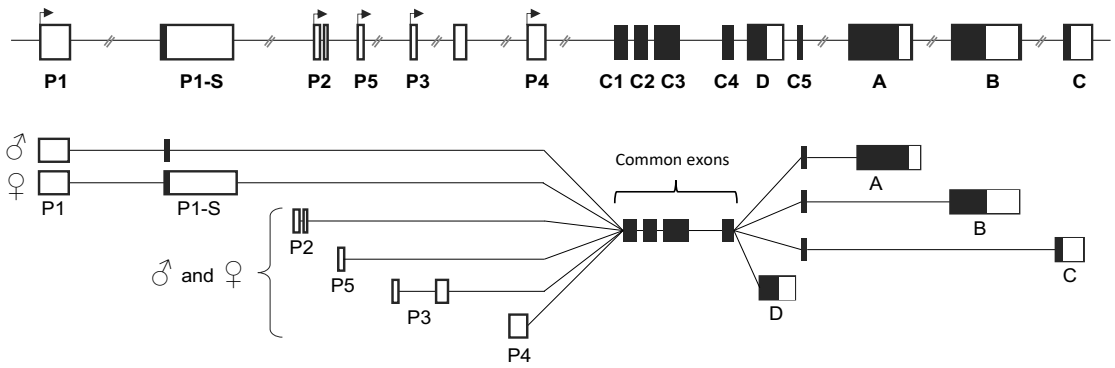

Figure 1-4 Organization of *fruitless* and its transcripts. The *fru* transcripts begin with one of five first exons (P1-P5) and undergo alternative splicing to produce five main classes of transcripts (Ito et al., 1996; Ryner et al., 1996). P1 transcripts undergo sex-specific splicing at the P1-S exon under the control of *tra* and *tra2* (Ryner et al., 1996). The transcripts from P2-P5 are not sex-specific and are present in both sexes. All *fru* transcripts share a set of common exons that encode the BTB (*Broad complex, Tramtrack* and *Bric-a-brac*) domains (Ito et al., 1996). Alternative splicing at the 3' end produces transcripts with one of four possible 3' exons (A-D) that encode zinc-finger DNA binding domains (Neville et al., 2014). Figure adapted from Stockinger et al. (2005).

The *fru* gene encodes a set of transcription factors with closely related BTB (*Broad complex, Tramtrack* and *Bric-a-brac*) zinc-finger domains (Ito et al., 1996; Ryner et al., 1996). The gene encodes at least 15 different transcript variants, generated through multiple exons and alternative splicing at both the 5' and 3' ends (Figure 1-4) (Ryner et al., 1996; Goodwin et al., 2000; Song et al., 2002). The *fru* transcripts begin with one of five first exons (P1-P5) that are alternatively spliced to produce five different transcript groups (Ito et al., 1996; Ryner et al., 1996). The P1 transcript is sex-specifically spliced at the S exon by the products of the *tra* and *tra-2* genes in females, causing the transcript to contain an early stop codon. The female specific transcripts (Fru^F) are detectable in the central nervous system of wild-type females, but due to the early stop codon they are not translated into functional proteins (Reviewed by Siwicki and Kravitz, 2009). The absence of *tra* and *tra-2* splicing in males results in the transcription of male specific *fruitless* transcripts (Fru^M) that lack this early stop codon and thus are translated into functional proteins (Hall, 1978; Ito et al., 1996; Ryner et al., 1996).

The *fru* locus was originally considered a "binary switch" that was necessary and sufficient for the development of male courtship behaviour, as well as male specific morphological traits (Usui-Aoki et al., 2000). Inducing female specific splicing of *fru* P1 in males abolished male courtship, while ectopic expression of male Fru^M proteins in females was sufficient to induce male courtship behaviour and the development of the MOL in females (Usui-Aoki et al., 2000; Demir and Dickson, 2005; Manoli et al., 2005;). However, a more recent study showed that Fru^M deficient males could acquire the ability to court when they are grouped with other flies (Pan and Baker, 2014). The study also showed that male specific Dsx^M protein is necessary for this ability to learn courtship from social interactions (Pan and Baker, 2014). This indicates that the previously postulated theory of *fruitless* as a master regulator and binary switch for controlling male courtship was premature, and it is more likely that *fruitless* is an important component in an elaborate gene network.

The pattern of *fruitless* P1 expression is sexually dimorphic with male specific Fru^M proteins detected in approximately 20 neural clusters composed of 1700 neurons in late larval and mid pupal stages (Lee et al., 2000). In adults, approximately 3% of CNS

neurons, as well as the neurons innervating the abdominal ganglion and the genitalia, express Fru^M. (Hall, 1978; Gailey and Hall, 1989; Usui-Aoki et al., 2000; Billeter, et al., 2006). In the adult female CNS, *fru* protein expression is limited to the developing ganglia in a pattern different from that of the male Fru^M proteins (Lee et al., 2000). However, the most well-characterized example of sexually dimorphic *fru* P1 expression is in a subset of interneurons known as the mAL (Kimura et al., 2005). In the male brain the mAL cluster is composed of 30 neurons, while cell death in females reduces the mAL cluster to five neurons (Kimura et al., 2005).

The other transcripts of *fru* (P2-P5) are not sex-specifically spliced, and are necessary for adult viability and external morphology (Anand et al., 2001; Ryner et al., 1996; Song et al., 2002). The P2, P3 and P4 transcripts are primarily expressed in the central nervous system (CNS) and are likely involved in the formation of axonal tracts in adults (Song et al., 2002). The non sex-specific *fru* proteins are distributed throughout the body in neural and non-neural tissues in both sexes, and are likely involved in the formation of axonal tracts in adults (Song et al., 2002).

The *fru* gene also undergoes alternative splicing at the 3' end resulting in five different isoforms of *fru* proteins with distinct C-terminal zinc finger (ZnF) domains. Three of these isoforms (FruA-C) are predominantly expressed in the central nervous system (Baker et al., 2001). Recently, *fru* proteins were shown to interact with the transcriptional co-factor *Bonus* (*bon*) to form a *fru-bon* complex and recruit two antagonistic chromatin modifying factors to HDAC1 and HP1 (Ito et al., 2012). This complex is involved in chromatin remodeling, and *fru* mediated regulation of gene expression at the neuronal level (Ito et al., 2012). Several putative downstream targets of *fru* have been identified, which include, *defective proboscis extension response* (*dpr*), *hunchback* (*hb*), *yellow* (*y*) and *takeout* (*to*) (Dauwalder et al., 2002; Drapeau et al., 2003; Goldman and Arbeitman, 2007; Dalton et al., 2013), but it is not clear if these genes directly interact with *fru.* A genome wide Chromatin Immunoprecipitation coupled to deep sequencing (ChIP-Seq) that was used to identify binding sites for *fru* found putative target genes involved in cellular processes. These gene functions included ion channel signaling, neuromuscular junction development, and neurotransmission (Vernes, 2014).

Male sexual behaviour has been the primary focus in studies on the function of *fru* (Ryner et al., 1996; Lee et al., 2000; Usui-Aoki et al., 2000; Anand et al., 2001; Demir and Dickson, 2005; Billeter et al., 2006b), and there is very little evidence for *fru's* role in female behaviour. However, *fru* has been linked to female receptivity, in conjunction with the genes *doublesex* and *retained* (Ditch et al., 2005; Shirangi et al., 2006). The *retained* gene acts as a constitutive female factor in both sexes: in females it acts with Dsx^F and feminizes neural pathways for reproductive behaviour, while suppressing intrinsic neural pathways for male sexual behaviours (Ditch et al., 2005). This is evident in *retn* mutant females, which exhibit male courtship behaviour but resist courtship from males in a manner observed in females with ectopic *fru*M expression (Ditch et al., 2005; Shirangi et al., 2006). This indicates that while *fru* does not appear to influence female behaviour directly, it likely interacts with other genes, regulates gene networks, or controls development of neural pathways that influence female mating behaviours.

1.9 Overview of dissertation

The primary goal of this dissertation is to confirm and characterize how the *fruitless* gene influences female mating behaviours and contributes to the behavioural isolation between *Drosophila melanogaster* and *D. simulans.* Through a series of genetic mapping and gene expression experiments, I test if *fruitless* is affecting species-specific rejection of *D. melanogaster* males by *D. simulans* females and whether any natural variation at this locus is contributing to the behavioural isolation between these species. In Chapter 2 I use genetic mapping to test several different types of transposable element disruptions in different areas of the *fru* locus, and precise deletions of *fru exons* to discern if a specific *fru* transcript or group of *fru* transcripts are responsible for species-specific rejection. I also use transposase-mediated remobilization of one of the transposable element disruptions in *fru* to rescue species-specific female receptivity, confirming *fru's* role in this trait. Furthermore, I test whether *fru* is affecting female rejection by acting through a specific sensory modality. In Chapter 3, I test precise disruptions in the 3' exons of *fru* to determine if *fru* transcripts with a specific 3' end are influencing species-specific female rejection. I then use the *GAL4-UAS* system to drive *fru* expression of 3' end variants in a *fru* mutant background to rescue female receptivity. In Chapter 4, I generate allele-

specific RNA interference (RNAi) strains. These strains can be used to knockdown specific *D. melanogaster* or *D. simulans fru* transcripts in particular tissues or neurons, allowing for the identification of the specific cells involved in species-specific female rejection behaviour.

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Chapter 2

2 The *fruitless* gene affects female receptivity and behavioural isolation between *D. melanogaster* and *D. simulans*

2.1 Introduction

Females typically have a greater investment than males into reproduction. Why? The reason is the value of their gametes and alternative strategies for maximizing fitness. Females have relatively few large eggs of high value, whereas males have many small sperm that are of individually low value (Bateman, 1948; Trivers, 1972; Clutton-Brock, 2007). As a consequence, females can maximize their fitness by choosing to mate with a few high-quality males and investing heavily into her offspring. The female strategy is therefore quality over quantity. Males, by contrast, can maximize their fitness through more-or-less indiscriminant matings with large numbers of females. The male strategy is therefore quantity over quality. As such females are expected to be much more choosy than are males when it comes to mating (Parker, 1983; Houde, 1987; Houle and Kondrashov, 2002).

Female discrimination of potential mates can act as a barrier to mating between species. This is especially true in species characterized by strong sexual selection which directly affects mating patterns in species expressing sexual traits and mating preferences that evolve rapidly and vary considerably between closely related taxa (Andersson and Simmons, 2006; Maan and Seehausen, 2011). For example, in species where indiscriminate males court females from other closely related species, they are usually rejected by these heterospecific females, which prevents hybridization between species (Wood and Ringo, 1980). Therefore, divergence in female preference for male mating traits can contribute to behavioural isolation between closely related species. Female mate preference has been shown to have a large genetic component through artificial selection experiments in the two-spot lady bird species *Adallia bipunctata* (Majerus et al., 1982). Since then several loci have been identified as candidate genes for natural variation in female preference both within (Finley et al., 1997; Suzuki et al., 1997;

Yamamoto et al., 1997; Juni and Yamamoto, 2009; Laturney and Moehring, 2012a) and between species (Campesan et al., 2001; Fitzpatrick et al., 2005; Kronforst et al., 2006; Moehring et al., 2006; Laturney and Moehring, 2012b), but only a few loci have been confirmed to affect species-specific female preference.

The *Drosophila* genus has multiple species that exhibit varying degrees of mating isolation, making it a commonly-used model for studying behavioural isolation (Laturney and Moehring, 2012a; Nanda and Singh, 2012). *Drosophila melanogaster* and *D. simulans* are two species that are behaviourally isolated. While *D. melanogaster* females mate at high frequencies with *D. melanogaster* males, *D. simulans* females reject courtship attempts from *D. melanogaster* males and do not mate (Barker, 1967; Carracedo et al., 2000). However, hybrid females from the cross between *D. melanogaster* females and *D. simulans* males (Sturtevant, 1920; Manning, 1959) will mate with *D. melanogaster* males (Davis et al., 1996). This lack of discrimination by hybrid females resembles *D. melanogaster* female behaviour, which suggests that *D. melanogaster* alleles affecting female receptivity are likely dominant, or semi-dominant, over the corresponding *D. simulans* non-receptive alleles.

D. melanogaster and *D. simulans* do not produce fertile hybrid offspring (Sturtevant, 1920; Lachaise et al., 1986). As a consequence, recombination mapping is not possible between these two species. Laturney and Moehring, (2012b) therefore used deficiency mapping to identify small regions of the third chromosome that influence species-specific female preference. This study utilized pre-existing *D. melanogaster* lines that are missing a small portion of their genome (a deficiency). These deficiencies were used to produce hybrids that contain one full set of both parents' genomes and one deficient region where only the *D. simulans* genome was present (Figure 2-1A). For deficiencies where hybrid females behaved like *D. simulans* by rejecting *D. melanogaster* males, the deficient region was further fine-mapped utilizing smaller deficient regions. Subsequent fine mapping within one of these regions identified a small genomic region containing a candidate gene: *fruitless* (*fru*; Figure 2-1B).

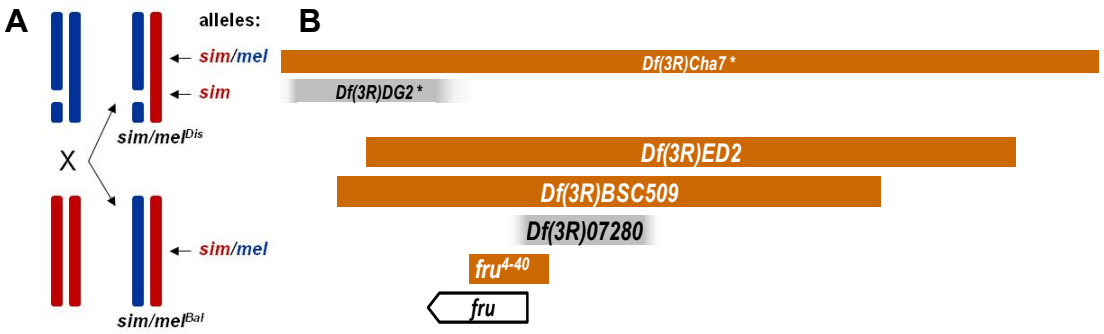

Figure 2-1 Deficiency mapping to identify candidate genes influencing female rejection of interspecific males. (A) Schematic of the 3rd chromosomes (blue and red lines) in deficiency mapping. *Drosophila. melanogaster* (*mel*, top left) females bearing a deficiency or disruption (broken blue line) are crossed to *D. simulans* (*sim*, bottom left; red lines) produce hybrids inheriting intact chromosomes from both species (bottom right; sim/mel^{Bal}) and hybrids inheriting a disruption in the *D. melanogaster* chromosome (top right; sim/mel^{Dis}). Gene(s) within this disrupted region are only expressed from the *D. simulans* homolog. **(B)** Deficiencies used to map female rejection of interspecific males. Rectangular bars represent deficiencies; blurred ends represent imprecisely known breakpoints; scale is approximate. The three deficiencies at top (marked with *) are from Laturney and Moehring (2012a). Orange is statistically significant ($P<0.05$); grey is not statistically significant. Arrowed box represents location and direction of *fru* gene, which is in inverse orientation.

The *fru* gene encodes a set of transcription factors generated via alternative exons at both the 5′ and 3′ ends (Figure 2-2; Ryner et al.,1996; Goodwin et al., 2000; Anand et al., 2001; Song et al., 2002). Transcripts begin with one of five first exons (P1-P5) and end with one of four final exons (A-D), with a central common region shared by all transcripts. The function of *fru* has been primarily studied in relation to the P1 transcript group's role in generating male courtship behaviour (Hall, 1978; Gailey and Hall, 1989; Ryner et al., 1996; Siwicki and Kravitz, 2009). P1 transcripts are sex-specifically spliced at the S exon by the products of the *transformer* and *transformer-2* genes to produce female specific transcripts (fru^F), while an absence of this splicing results in male-specific *fru* transcripts (fru^M ; Figure 2-2; Billeter et al., 2006). fru^F transcripts are not translated into proteins, but are detectable in the central nervous system of wild-type

females (Usui-Aoki et al., 2000). The proteins translated from fru^M are expressed in approximately 3% of the neurons in the central nervous system (Goodwin et al., 2000; Lee et al., 2000) as well as the neurons innervating the abdominal ganglion and the genitalia (Baker et al., 2001; Billeter et al., 2006). They are also necessary for the development of the male specific Muscle of Lawrence (MOL) and normal male courtship (Usui-Aoki et al., 2000).

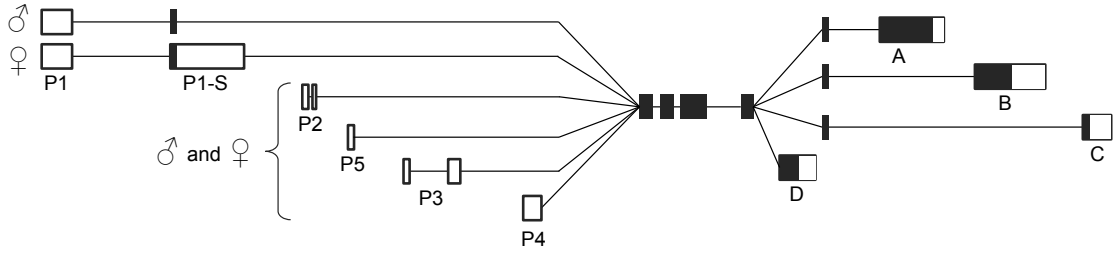

Figure 2-2 Representation of *fru* transcripts. The *fru* transcripts begin with one of five first exons (P1-P5) and undergo alternative splicing to produce five main classes of transcripts (Ito et al., 1996; Ryner et al., 1996). P1 transcripts undergo sex-specific splicing at the P1-S exon under the control of *tra* and *tra2* (Ryner et al., 1996). The transcripts from P2-P5 are not sex-specific and are present in both sexes. All *fru* transcripts share a set of common exons (C1-C5) that encode the BTB domains (Ito et al., 1996). Alternative splicing at the 3' end produces transcripts with one of four possible 3' exons (A-D) that encode zinc-finger DNA binding domains (Neville et al., 2014). Boxes are exons, black boxes are coding. Adapted from Stockinger et al. (2005)

The *fru* locus plays additional roles beyond those in male courtship and sex determination. While far less studied than the P1 transcripts, a subset of the sex-nonspecifically spliced transcripts (P2-P5) are necessary for adult viability and external morphology (Ryner et al., 1996; Anand et al., 2001; Song et al., 2002). The P2 transcript is most strongly expressed in the eye, while the P3 and P4 transcripts are primarily expressed in the developing CNS and are likely involved in the formation of axonal tracts (Song et al., 2002; Leader et al., 2017;). While the P1 transcript appears to have no role in female mating behaviour (Baker et al., 2001), none of the other transcripts have been assayed for their effect on female preference.

If *fruitless* is indeed influencing species-specific female preference, it might be doing so by affecting females' perception of male courtship signals. *Drosophila* male courtship involves multimodal signals exchanged through a series of stereotypic courtship steps. During this exchange, the female assesses the male based on these signals and chooses to either copulate with or reject the male. The primary cues that have historically been examined are the male auditory cue of courtship song produced by wing vibrations, which differs between these two species (Ewing and Bennet-Clark, 1968; Moulin et al., 2001, 2004), and the chemical pheromonal cues, which also differ between these two species (Cobb and Jallon, 1990). Removal or alteration of either of these individual components of male courtship does not cause females to become fully receptive to heterospecific males (Ritchie et al., 1999; Tomaru et al., 2000), presumably because all of the other courtship components are still interspecific, and a single negative courtship signal can be sufficient to induce female rejection. In the context of hybrid females bearing a *fru* disruption, however, it is possible that the rejection induced by the *fruitless* gene may result from the processing of a single component of courtship, and removal of that component might induce *D. melanogaster*-like receptivity in these females.

In this chapter, I investigate the potential role that *fruitless* plays in species-specific female preference, and whether a specific *fruitless* transcript is affecting this trait. I also attempt to rescue female receptivity to males from different species through the removal of a disruption in *fru.* I perform RT-PCR to determine if the expression pattern of *fruitless* transcripts is affected by various *fru* disruptions. Lastly, I investigate whether the *fruitless* gene is affecting species-specific female preference by acting through specific male courtship cues or through females' assessment of male courtship signals.

2.2 Methods

***Drosophila* Strains:** Flies were maintained on standard food medium (Bloomington *Drosophila* Stock Center) under a 14:10 light:dark cycle at 24ºC and approximately 80% relative humidity. All *Drosophila melanogaster* disruption stocks are listed in Table 2-1. Deficiencies and gene disruptions were maintained over 3rd chromosome balancers *TM3* or *TM6C*, which contain a visible phenotypic marker and serial inversions preventing recovery of recombinant offspring between the homologous 3rd chromosomes. Unless

otherwise noted, all stocks were obtained from the Bloomington *Drosophila* Stock Center (Bloomington, Indiana), as were line #3703 ($w^{1118}/Dp(1;Y)y^{+}$; CyO/nub^{1} b^{1} sna^{Sco} lt^{1} stw^{3}; $MKRS/TM6B$, Tb^{1}) and the *Minos* transposase stock (w^{1118}; $\underline{sna^{Sco}}/\underline{SM6a}$, $\underline{P\{hsILMiT\}2.4}$). The *fru*-GAL4 and fru^{4-40} lines were provided by Dr. Barbara Taylor. The *D. melanogaster* stock with GFP-tagged sperm (*w; P{w8, ProtA-EGFP,* w^{+}*}19B(3)*) was provided by Dr. John Belote. Wild-type *D. melanogaster* BJS is an isofemale line collected by Dr. Brent Sinclair. Wild-type *D. simulans* Florida City (FC) was provided by Dr. Jerry Coyne (collected from Florida City, Florida; Coyne, 1989); wild-type *D. simulans* 199 (stock # 14021-0251.199; collected from Nanyuki, Kenya) and wild-type *D. simulans* 216 (stock # 14021-0251.216; collected from Winters, California) were obtained from the *Drosophila* Species Stock Center (San Diego, California).

Mating Assays: Five to six virgin *D. melanogaster* females bearing a disruption over a balancer (mel^{Dis}/mel^{Bal}) were aged 5-7 days then crossed with 25-30 *D. simulans* FC or 3-4 *D. melanogaster* BJS non-virgin 5-10 day old males to produce F1 hybrid sim/mel^{Dis} and sim/mel^{Bal} or mel/mel^{Dis} and mel/mel^{Bal} offspring, respectively. Virgin F1 females of the four genotypes produced above were paired with a 5-7 day old virgin *D. melanogaster* GFP-sperm male in a no-choice mating assay. Assays were carried out at 24ºC, ~50% relative humidity, and 1-2 hours of "lights on" in 30mL plastic vials containing approximately 5 mL of standard food medium. All four genotypes (sim/mel^{Dis}, sim/mel^{Bal}, mel/mel^{Dis} and mel/mel^{Bal}) were assayed in equal numbers on any given assay day to account for uncontrolled environmental effects that could influence mating activity (Austin et al., 2014). Flies were observed for 1 hour ("1 hour mating assay") and scored for latency to courtship and latency to copulation. From these measures, the proportion that copulated out of those that were courted was calculated.

Assays involving F1 hybrid sim/mel^{Dis} and sim/mel^{Bal} females were left within the vials for an additional 24 hours ("24 hour mating assay"), at which time the female was decapitated and her reproductive tract dissected. The tract was imaged using a compound fluorescent microscope and scoring for the presence or absence of fluorescently-labelled sperm to infer copulation occurrence. The number of females that copulated out of those

that were courted was compared using logistic regression ($\alpha < 0.05$) with the independent variables of species (*mel/mel* or *sim/mel*) and genotype (*Dis* or *Bal*) and the dependent variable of whether copulation occurred after courtship (*yes* or *no*). Logistic regression compares the likelihood ratios between expected counts and the observed counts for each category; the effect of interest here is the species × genotype interaction term. I considered a result biologically significant only if the sim/mel^{Dis} females had reduced copulation compared to sim/mel^{Bal} (inter-species control), after the values are corrected for any effects of the balancer or disruption chromosomes themselves, as determined using mel/mel^{Dis} and mel/mel^{Bal} (intra-species controls).

To test whether the reduction in receptivity due to *fru* occurs across *D. simulans* or is specific to strain FC, the above assays were repeated by Dr. Ryan Calhoun for $fru^{MI01850}$ mel^{Dis}/mel^{Bal} crossed to *D. simulans* stock 199 and *D. simulans* stock 216. As above, I compared the four genotypes for each cross using a logistic regression. Likewise, to test whether these females experience a general disruption in female receptivity, Ipaired fru^{4-40}, fru^{GAL4} and $fru^{KG00116}$ sim/mel^{Dis} and sim/mel^{Bal} females in no-choice mating assays as above, but using *D. simulans* FC males instead of *D. melanogaster* males. As the sperm are not fluorescently labelled, I scored for the presence or absence of sperm using a compound microscope. The presence of sperm was used as a proxy for copulation occurrence. The proportion of females that mated for sim/mel^{Dis} was compared to those for sim/mel^{Bal} using a Z-test ($\alpha < 0.05$).

Molecular confirmation of fru transcripts: For each disruption line, RNA was extracted from 20 adult females (5-7 day old) that are homozygous for the *fru* disruption. The $fru^{MI05459}$ line is homozygous lethal, and to obtain homozygous flies for RT-PCR, $fru^{MI05459}$females were crossed with stock #3703 to create the genotype $y^1\ w^*;\ fru^{MI05459}/TM6B,\ Tb^1$. Late stage non-$Tb^1$ pupae, which are homozygous for the disruption, were collected, and adult females were dissected out of the pupa casing (Anand et al., 2001). Total RNA was extracted using the TRIzol plus Purelink RNA purification kit (Thermofisher Cat# A33254). RNA was quantified using a Nanophotometer P300 (Implen, Inc.) and 2 ug of total RNA was used for cDNA synthesis using Maxima First Strand cDNA Synthesis Kit with DsDNAase

(Thermofisher, Cat# K1671) using oligo dTs. RT-PCR was performed to check for presence of *fru* P1, P2, P5, P3 and P4 transcripts using a forward primer within each of the respective exons and a reverse primer within the common region of *fru*. *RpL32* was used as a control to compare gene expression levels (Appendix: Figure S2-1). RT-PCR was also performed for all transcripts in *D. simulans* FC, to confirm that they are present (data not shown). All primers are listed in Appendix: Table S2-1.

Excision of MiMIC insertion: The *Minos* mediated Integration Cassette (MiMIC) in $fru^{MI05459}$ was excised by crossing to a *Minos* transposase stock. Balancers and visible markers from line #3703 were integrated, allowing for the generation of flies with the genotype $y^1\ w^*;$ SM6a, P{hsILMiT}2.4/CyO; $Mi\{MIC\ y^+\}fru^{MI05459}/TM6B,\ Tb^1$. To generate flies with the *Minos* element removed, males from this stock were crossed with $y^1\ w^*;$ $CyO/nub^1\ b^1\ sna^{Sco}\ lt^1\ stw^3;\ MKRS/TM6B,\ Tb^1$ virgin females (Arcà et al., 1997) and larvae of this genotype were heat shocked for 1 hour at 37ºC on days 3, 5 and 7 (Nagarkar-Jaiswal et al., 2015). After maturing to adulthood, heat-shocked males were placed in single-pair matings with $y^1\ w^*;$ $CyO/nub^1\ b^1\ sna^{Sco}\ lt^1\ stw^3;\ MKRS/TM6B,\ Tb^1$ virgin females. After five days to allow for offspring production, the males were screened for the excision of the MiMIC insertion by PCR genotyping using primers flanking the MiMIC insertion site. Offspring produced from individuals with excisions were crossed together to create a stable stock with genotype $y^1\ w^*;\ [excised:\ Mi\{MIC\ y+\}fruMI05459]$. The excisions were confirmed by sequencing the region flanking the MiMIC insertion site (See Appendix: Table S2-1; Figure S2-2).

Five to six virgin females from the clean excision stock and virgin females from the original $fru^{MI05459}$ *Minos* disruption stock were separately crossed with 25-30 non-virgin 5-10 day old *D. simulans* FC males to create F1 hybrid sim/mel^{Dis+}, sim/mel^{Dis}, and sim/mel^{Bal} females, where *Dis+* indicates the excised *Minos* disruption. F1 hybrid females were aged 5-7 days and then paired separately with 5-7 day old virgin *D. melanogaster* GFP-sperm males in no choice mating assays. Copulation success was scored in a 24 hour assay, as above, and the proportions copulated were compared using Z-tests ($\alpha < 0.05$).

Targeted removal of *fruitless* P1 and P2 promoter sequences: CRISPR-Cas9 genome editing was used to remove P1 promoter (first exon) sequences in the *fru* locus generating line $fru^{\Delta P1}$ (Appendix: Figure S2-3A). Two fragments with homology to the *fru* genomic regions on either side of the P1 promoter were cloned using Gibson Assembly Master Mix (New England Biolabs) with primers for 5' Fragment: G1_5f and G1_5r and 3' Fragment: G1_3f and G1_3r (see Appendix: Table S2-2), into the targeting vector pDsRed-attP (a gift from Melissa Harrison & Kate O'Connor-Giles & Jill Wildonger; Addgene plasmid # 51019) digested with *Xho*I and *Not*I. gRNA expressing constructs pCFD3-Fru5_1 and pCFD3-Fru3_3 were generated in the vector pCFD3-dU6:3gRNA (a gift from Simon Bullock Addgene plasmid #49410) against target regions Fru5_1 and Fru3_3 (see Appendix: Table S2-2), respectively, as described in Port et al. (2014). The resulting constructs were co-injected into the strain *vas*-Cas9.RFP^- (Bloomington stock #55821), progeny were screened for $DsRed^+$ expression in the eye. Seven lines were identified, all of which mapped to the 3rd chromosome. PCR and sequencing were used to confirm the deletion of P1 sequences, followed by immunohistochemistry analyses to confirm the absence of male-specific Fru^M expression (data not shown).

The P2 promoter (first exon) in the *fru* locus was targeted by ends-out homologous recombination as previously described (Gong and Golic, 2004), generating line $fru^{\Delta P2}$ (Appendix: Figure S2-3B). Two fragments with homology to *fru* genomic regions on either side of the P2 promoter were cloned sequentially into the targeting vector pP{W25} digested first with *Bsi*WI and *Asc*I, followed by *Kpn*I and *Not*I, using primers: P2_5f and P2_5r for the 5' homology arm and primers P2_3f and P2_3r for the 3' homology arm (see Appendix: Table S2-2). The resulting construct was injected into a w^{1118} strain. One of the ensuing second chromosome transformant lines was crossed (2000 virgin females) to males of the genotype *y,w/Y,hs-hid; Sco,hs-I-SceI,hs-FLP/CyO*; first instar larvae were heat shocked for 1.5 hrs at 38°C on the 3rd day following the cross and again on the following day for 1hr. The following fly stocks were used to identify and balance all third chromosomal $white^+$ recombinants: *y,w; ey-FLP, y,w,ey-FLP; Pin/CyO, y,w,ey-FLP;;Ly/TM3,Sb*. Approximately 100,000 flies were screened, and 16 independent targeted recombination events were recovered. Cre/*loxP* recombination (Bloomington stock #851) was used to remove $white^+$. Finally, PCR, sequencing, and

Southern blot analysis were used to confirm the predicted recombination events (data not shown).

***fruitless* mutant fertility analysis:** Two independent lines for $fru^{\Delta P1}$ ($fru^{\Delta P1.1}$ and $fru^{\Delta P1.2}$) and one line for $fru^{\Delta P2}$ were backcrossed eight times to the wild-type strain *Canton-S*. Fertility was measured by placing 3-day old males or females individually in food vials containing three wild-type virgins of the opposite sex. All vials were scored for presence of progeny after 7 days (Appendix: Figure S2-3C, D). Percentage fertility is determined by the proportion of flies that produced viable progeny.

Sensory modality assays: Sensory modality assays were used to test whether removal of male courtship song, female auditory perception, and substrate-borne vibrations, would rescue the hybrid female rejection phenotype that was produced by unmasking the *D. simulans* allele of *fru*. In all sensory modality assays, 5-7 day old virgin females from the aforementioned interspecific crosses were paired with 5-7 day old virgin *D. melanogaster* GFP-sperm males in 24-hour no-choice mating assays, as described above.

To test the effect of courtship song on species specific female preference, males were anaesthetized and de-winged 48 hours before the mating assays by clipping their wings (wing-) using dissection tweezers immediately distal to the hinge (Krstic et al., 2009). Males were given 48 hours to recover then paired in no-choice mating assays with $fru^{4\text{-}40}$, fru^{GAL4}, and $fru^{MI05459}$ sim/mel^{Dis} females. Control males were anaesthetized at the same time but were not de-winged (wing+). Copulation occurrence for wing+ *vs.* wing- males was compared using a Z-test ($\alpha < 0.05$).

To test whether *fruitless* is acting on female perception of olfactory or auditory cues, 48 hours before the assay virgin fru^{GAL4} sim/mel^{Dis} females were placed under CO_2 anesthesia and either left unaltered (ant+) or dissection tweezers were used to remove the last two segments of the females' antennae (ant-), which includes the aristae. Females were given 48 hours to recover, then paired with males in no-choice mating assays with these females. Copulation occurrence of ant+ *vs.* ant- females was compared using a Z-test ($\alpha < 0.05$).

To test whether substrate vibrations had an effect in female preference, 5-7 day old virgin hybrid fru^{GAL4} sim/mel^{Dis} and corresponding sim/mel^{Bal} females were paired with 5-7 day old virgin *D. melanogaster* GFP sperm males in a Plexiglass mating arena (Dierick, 2007) placed on a granite base. The base, if left bare, does not allow transmission of the substrate vibrations produced by small insects (Elias et al., 2004) The mating arena was coated with Insect-a-Slip Insect Barrier from BioQuip Products (Cat# 2871C), to prevent flies from climbing the walls or ceiling of the arena. For half of the chambers in the arena, the granite base was coated with 1% agarose gel approximately 2mm thick to add a substrate for the control assays. The mating assays were performed for 24 hours, as described above. A four-way comparison of copulation success of sim/mel^{Dis} and sim/mel^{Bal} hybrid females with or without substrate were compared using logistic regression.

2.3 Results

To confirm whether *fru* had an effect on female preference, I used the same conceptual approach as deficiency mapping but using the small deletion fru^{4-40} known to eliminate sex-specific *fru* function (Anand et al., 2001); and two disruptions known to affect sex-specific *fru* expression (fru^{GAL4} and fru^{NP0021}; Anand et al., 2001; Kimura et al., 2008, 2005; Lee et al., 2000; Stockinger et al., 2005); and three transposable-element insertions in *fru* that have yet to be fully characterized but may disrupt *fru* function ($fru^{MI01850}$, $fru^{KG00116}$, and $fru^{MI05459}$ (Bellen et al., 2004; Gramates et al., 2016; Venken et al., 2011). When there is an insertion in the *D. melanogaster* allele of *fru* in an interspecies hybrid, so that only the *D. simulans fru* allele is likely functional (sim/mel^{Dis}), female receptivity towards *D. melanogaster* males was significantly reduced compared to controls in five of the six lines that I tested (Figure 2-3; Table 2-1). Additionally, I matched the *fru* allele being expressed to the species of the male in the assay by pairing sim/mel^{Dis} females with *D. simulans* males to verify whether that the effect of *fru* on female receptivity is species-specific. Hybrid Females bearing any of the three *melanogaster fru* disruptions that I assayed mated similarly to the controls lacking this disruption (sim/mel^{Bal}) when paired with *D. simulans* males (Table 2-2).

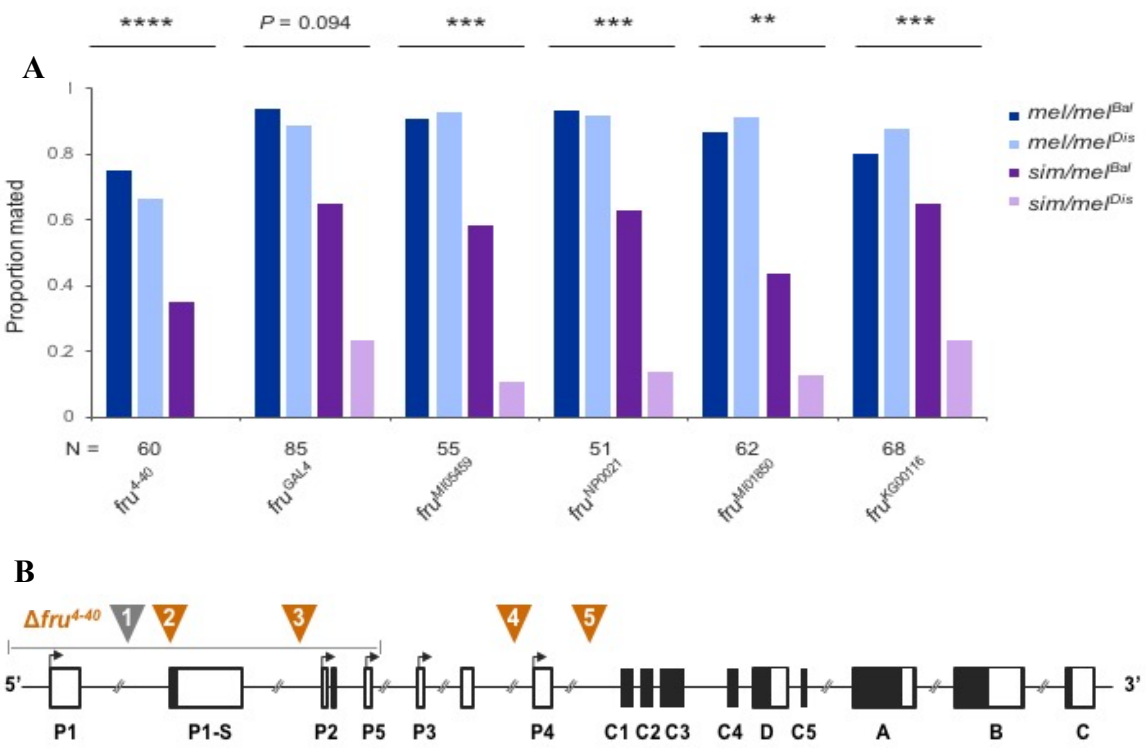

Figure 2-3 Behavioural assays with *fruitless* disruptions paired with *D. melanogaster* males. (A) Proportion of females mated when paired with *D. melanogaster* males for control pure species females without a disruption (*mel/mel*^{Bal}), with a disruption (*mel/mel*^{Dis}), and hybrid females without a disruption (*sim/mel*^{Bal}), when compared to hybrid females with a disruption (*sim/mel*^{Dis}). Comparisons where *sim/mel*^{Dis} females have a significant reduction in mating have *P*-values shown in bold; N is listed for each genotype within the group. The proportion of matings were compared using logistic regression. Results were considered biologically significant only if the *sim/mel*^{Dis} females had reduced copulation compared to *sim/mel*^{Bal}, after values are corrected for effects of the balancer or disruption, determined by the *mel/mel*^{Dis} and *mel/mel*^{Bal} controls. **(B)** The location of *fru* disruptions within the *fru* locus shown 5' – 3' to represent the *fru* P1-P5 first exons, common exons C1-C5, and 3' exons A-D; boxes are exons, black boxes are coding. The relative location of transposable element insertions are represented by numbered inverted triangles: 1=*fru*^{GAL4}, 2=*fru*^{NP0021}, 3=*fru*^{MI05459}, 4=*fru*^{MI01850}, 5=*fru*^{KG00116}; the dashed line represents the *fru*$^{4-40}$ deletion. Orange is statistically significant ($P<0.05$); grey is not statistically significant. Image not to scale.

Table 2-1 Behavioural assays with *fruitless* disruption lines. Females were paired with *D. melanogaster* males. The number of courtships that occurred within 1 hour (Court) as well as the number of copulations in 1 hour (1h Cop) were recorded; hybrid females were also assessed for copulation occurrence after 24 hours (24h), after all females were presumed to have experienced courtship. Data analyzed by logistic regression; the interaction term of species (hybrid vs. mel) x genotype (Dis vs. Bal) determines significance. P-value is shown. D.f.=1

				sim/mel^{Bal}			*sim/mel*^{Dis}			*mel/mel*^{Bal}		*mel/mel*^{Dis}		
Name	***D. melanogaster Dis/Bal* genotype used to make hybrid females**	**Replicate**	**N**	**Court**	**1h Cop**	**24h Cop**	**Court**	**1h Cop**	**24h Cop**	**Court**	**1h Cop**	**Court**	**1h Cop**	***P*-value**
fru$^{4-40}$	*w**; *Df(3R)fru*$^{4-40}$/*TM3, P[actin-GFP] Sb*^{1} *Ser*^{1}	1	30	9	0	10	14	0	0	12	8	17	10	0.0068
		2	30	16	0	11	17	0	0	12	10	16	12	0.0013
		1+2												<0.0001
fru^{GAL4}	*w**; *TI{GAL4}fru*$^{GAL4.P1.D}$ / *TM3, Sb*^{1}	1	34	30	23	23	23	7	7	32	30	30	26	0.2579
		2	51	49	32	32	47	13	13	46	43	50	45	0.2107
		1+2												0.0938
fru^{MI05459}	*y*^{1} *w**; *Mi{MIC}fru*^{MI05459} / *TM3, Sb*^{1} *Ser*^{1}	1	27	22	14	14	23	2	2	27	24	27	25	0.0127
		2	28	27	18	18	25	4	4	28	26	28	26	0.0604
		1+2												0.0020
fru^{NP0021}	*w*^{1118}; *P{GawB}fru*^{NP0021} / *TM3, Sb*^{1}	1	21	19	16	16	18	2	2	19	18	20	17	0.1660
		2	30	27	3	16	27	4	5	27	25	28	27	0.0637
		1+2												0.0323
fru^{MI01850}	*y*^{1} *w**; *Mi{MIC}fru*^{MI01850} / *TM3, Sb*^{1} *Ser*^{1}	1	30	13	0	9	14	0	1	15	13	17	16	0.0292
		2	32	12	2	18	15	0	7	15	13	18	16	0.1577
		1+2												0.0209
fru^{KG00116}	*y*^{1} *w*^{67c23}; *ry*^{506} *P{SUPor-P}fru*^{KG00116} / *TM3, Sb*^{1}	1	38	14	1	23	23	3	8	24	22	25	22	0.2337
		2	30	17	2	21	18	2	8	21	14	16	14	0.0016
		1+2												0.0007
fruΔP1	*Fru P1 (Deletion), stock #1 / TM3, Sb*^{1} *Ser*^{1}	1	27	22	5	12	25	2	13	25	25	26	26	1.0000
fruΔP1	*Fru P1 (Deletion), stock #2 / TM3, Sb*^{1} *Ser*^{1}	1	29	23	6	15	28	4	12	27	26	29	24	0.2743
fruΔP2	*Fru P2 (Deletion) / TM3, Sb*^{1} *Ser*^{1}	1	41	38	19	29	35	1	9	27	24	26	25	0.0060

Table 2-2 Behavioural assays with *fruitless* disruption lines paired with *D. simulans* males. Hybrid females were paired with *D. simulans* FC males in 24 hour mating assay. Hybrid females were assessed for copulation occurrence after 24 hours (24h), after all females were presumed to have experienced courtship. Data analyzed by logistic regression; the interaction term of species (hybrid vs. mel) x genotype (Dis vs. Bal) P-value is shown. D.f. = 1.

D. melanogaster Dis/Bal genotype used to make hybrid females	N	*sim/mel*^{Bal} 24h Cop	*sim/mel*^{Dis} 24h Cop	Z-value	*P*-value
w^{*}; Df(3R)$fru^{4\text{-}40}$/TM3, P[actin-GFP] Sb^{1} Ser^{1}	19	8	12	-1.300	0.2101
w^{*}; TI{GAL4}$fru^{GAL4.P1.D}$ / TM3, Sb^{1}	30	15	13	0.518	0.6087
y^{1} w^{67c23}; ry^{506} P{SUPor-P}$fru^{KG00116}$ / TM3, Sb^{1}	20	8	6	0.663	0.5153

If *fru* is indeed involved in species-specific female preference behaviour, *D. melanogaster*-like female preference behaviour should be rescued after excising the transposable element insertion from one of the *fru* disruption lines. I chose to excise the $fru^{MI05459}$ *Minos* element insertion as it showed a strong effect on female preference (Figure 2-3A; Table 2-1). The $fru^{MI05459}$ insertion disrupts at least some *fru* transcripts, as it reduces transcription of P2 and eliminates transcription of P5 (Figure 2-4A) and $fru^{MI05459}/fru^{4\text{-}40}$ males are behaviourally sterile (Table 2-3) and $fru^{MI05459}/fru^{sat15}$ offspring are viable (25% expected, 24% seen: 86/357). After excision, sequencing and RT-PCR to confirm a clean removal of the *Minos* element (Figure 2-4A, Appendix: Figure S2-2), I assayed for female receptivity (Figure 2-4B). Hybrid females with the excised *Minos* element mated significantly more than hybrids bearing the disruption (P<0.0001, Z=4.21) and had only a slight non-significant reduction in mating frequency compared to control hybrids in which *fru* is intact (P=0.0549, Z=1.92).

To identify which *fru* transcript may be affecting female rejection behaviour, I first assessed the presence or absence of P1-P5 transcripts in females for five of the disruption lines I tested using RT-PCR (Appendix: Figure S2-1); $fru^{4\text{-}40}$ was previously shown to disrupt P1 and P2 transcripts and was not tested using RT-PCR (Anand et al., 2001). While the presence of *fru* transcript does not indicate functional transcript (Goodwin et al., 2000), most of the lines I tested had P1, P3 and P4 transcripts present, while several had reduced or absent P2 or P5 (Appendix: Figure S2-1).

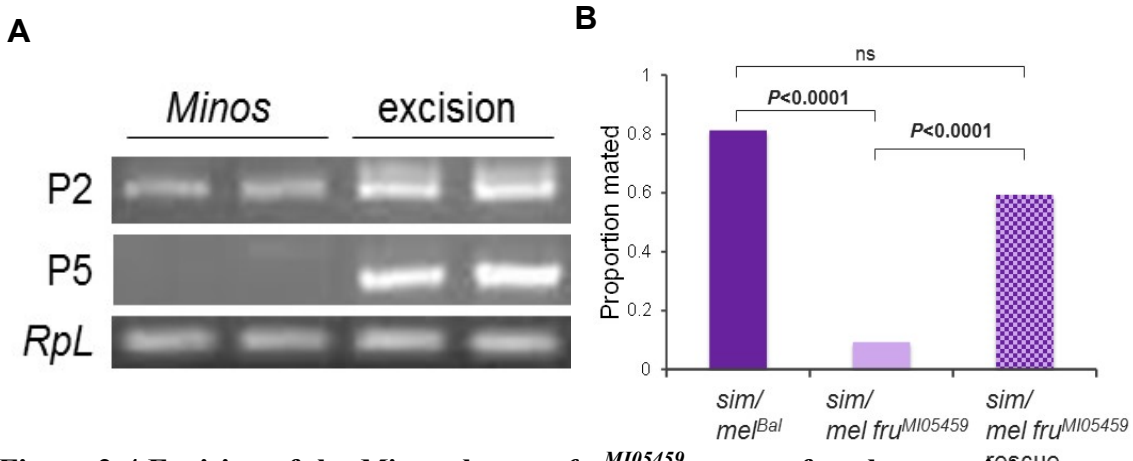

Figure 2-4 Excision of the *Minos* element *fru*^{MI05459} rescues female receptivity to inter-species males (A) Transcript presence in flies homozygous for the *Minos* insertion *fru*^{MI05459} compared to flies that have had the *Minos* element excised. The housekeeping gene *RpL32* is used as a control. Each sample was done with a biological replicate. **(B)** Proportion of matings in hybrid females with the *Minos* element excised, compared to hybrids with or without the *Minos* element (controls). Female receptivity is rescued when the *Minos* element within *fru*^{MI05459} is removed (N=32).

Table 2-3 Behavioural assays with males bearing the *fru*^{MI05459} insertion over *fru*$^{4-40}$ paired with *D. melanogaster* females. Courtship and copulation proportion of *fru*^{MI05459}/*fru*$^{4-40}$ males with *D. melanogaster* females in 1-hour mating assays. The *fru*^{MI05459}/*fru*$^{4-40}$ males are behaviourally sterile.

D. melanogaster male	N	Courtship latency (sec)	Proportion courted	Proportion copulated
w* *y*^{1} ; *fru*^{MI05459} / *Df(3R)fru*$^{4-40}$	22	N/A	0	0
Canton S	22	160s (± 320s st. dev.)	1.0	1.0

I then tested precise deletions of either the *D. melanogaster fru* P1 first exon or *fru* P2 exon (*fru*$^{\Delta P1}$ and *fru*$^{\Delta P2}$, respectively) to determine if the transcripts from one of these exons are affecting interspecies female rejection. These precise deletions were generated, verified and assayed for fertility by a Moehring Lab collaborator, Dr. Megan Goodwin. Hybrid females bearing a *melanogaster* P1 or P2 disruption are not courted less than control females by *D. melanogaster* males, as there is no significant difference in time

until a male initiates courtship (N=32, P=0.867, F=3.094) or total time the male spends courting (N=32, P=0.254, F=1.392; Table 4). Hybrid females bearing $fru^{\Delta P1}$, and thus only expressing *D. simulans* P1 alleles, did not show reduced receptivity towards *D. melanogaster* males. In contrast, $fru^{\Delta P2}$ hybrid females that only express *D. simulans* P2 alleles show reduced mating with *D. melanogaster* males (Figure 2-5A, B; Table 2-1).

I also tested whether the loss of P1 or P2 affects within-species female preference by assaying for female receptivity in pure species pairings. Male *D. melanogaster* court $fru^{\Delta P1}$ and $fru^{\Delta P2}$ females for a similar amount of time as *Canton*-S control females (N=30, P=0.094, F=3.101) but there is a difference in the time it takes for males to initiate courtship (N=30, P=0.0002, F=3.103; Table 2-4). In pairwise comparisons, the latency to courtship is shorter for $fru^{\Delta P1}$ (N=30, P=0.00028, t = 1.672) and $fru^{\Delta P2}$ (N=30, P=0.0028, t = 1.672) females compared to the control. Additionally, *D. melanogaster* females homozygous for the $fru^{\Delta P2}$ deletion exhibit reduced mating with *D. melanogaster* males compared to $fru^{\Delta P1}$ and *Canton*-S females (N=30, P=0.01, Z=2.334; Table 2-5).

Table 2-4 Courtship assays with hybrid females bearing $fru^{\Delta P1}$ or $fru^{\Delta P2}$ deletions paired with *D. melanogaster* males. Time until the initiation of courtship (courtship latency) and the total time a wildtype *D. melanogaster* male spent courting a hybrid female within a 1-hour courtship assay.

D. melanogaster genotype used to make hybrid females	N	Courtship latency (sec)	Total courtship time (sec)
Fru P1 (Deletion), stock #1 / TM3, Sb ^{1} Ser ^{1}	**32**	71.5s (± 65s st. dev.)	3013.2s (± 862s st. dev.)
Fru P2 (Deletion) / TM3, Sb ^{1} Ser ^{1}	**32**	87s (± 89s st. dev.)	2633.2s (± 990s st. dev.)
Canton S	**32**	69.5s (± 64s st. dev.)	2666s (± 1154s st. dev.)

When paired with *D. simulans* males in a five-day mating assay period, there was no difference in copulation frequency among the deletions and control (N=15, P=0.154, Z=1.017), but all three groups of females show very low mating in this interspecies assay. As I cannot be certain that a P2 deletion does not also subtly affect P5 expression, and to determine if a P2 transcript could plausibly affect behaviour via the female brain, I used

RT-PCR to confirm that the P2 transcript, but not the P5 transcript, is expressed in female brains (Figure 2-6).

Table 2-5 Courtship assays with *D. melanogaster* females bearing $fru^{\Delta P1}$ or $fru^{\Delta P2}$ deletions paired with *D. melanogaster* males. Time until the initiation of courtship (courtship latency), total time spent courting, and proportion that copulated when pairing a wild-type *D. melanogaster* male with a *D. melanogaster* female in a 1-hour assay.

D. melanogaster females	N	Courtship latency (sec)	Total courtship time (sec)	Propotion copulated
Fru P1 (Deletion), stock #1	**30**	62s (± 61s st. dev.)	310.3s (± 518s st. dev.)	0.966666667
Fru P2 (Deletion)	**30**	88.3s (± 59s st. dev.)	756.8s (± 1076s st. dev.)	0.7
Canton S	**30**	178s (± 163 s st. dev.)	472.5s (± 516s st. dev.)	0.966666667

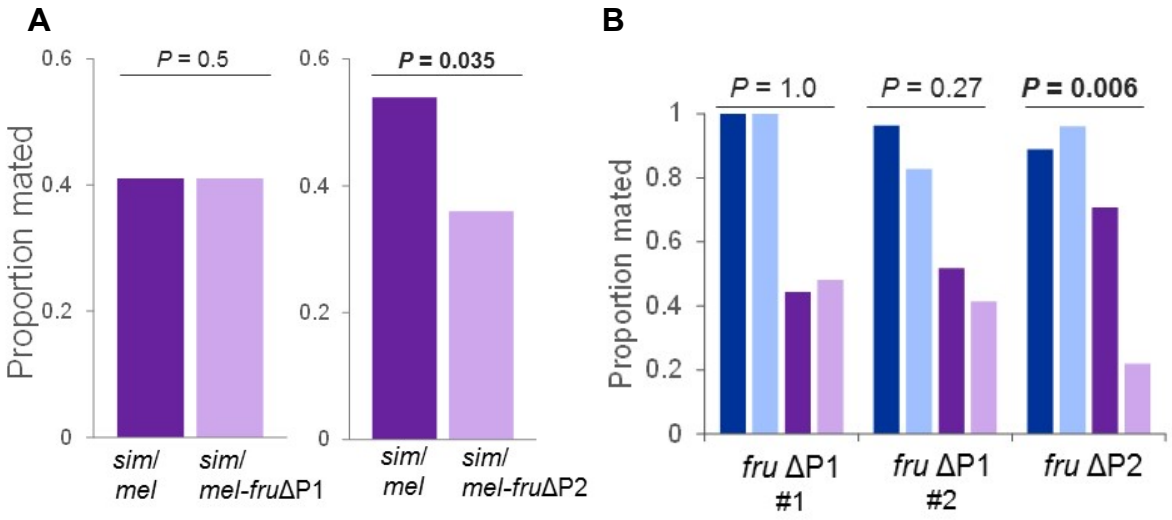

Figure 2-5 Behavioural assays with hybrid females bearing $fru^{\Delta P1}$ or $fru^{\Delta P2}$ deletions paired with *D. melanogaster* males. (A) Proportion of matings for hybrid females bearing $fru^{\Delta P1}$ (independent deletions #1 and #2) or $fru^{\Delta P2}$ targeted deletions of the *D. melanogaster* allele (the *D. simulans* allele is intact). Proportion of mating for $fru^{\Delta P1}$ (#1, N=39) or $fru^{\Delta P2}$ (N=50) hybrid females, compared to a hybrid Canton S/*simulans* controls with a *Z* test. **(B)** $fru^{\Delta P1}$ (#1, N=27; #2, N=29) and $fru^{\Delta P2}$ (N=41) disruptions in hybrid females (sim/mel^{Dis}; light purple) compared to hybrid females without a disruption ($siml/mel^{Bal}$; dark purple), pure species females with (mel/mel^{Dis}; light blue) and without (mel/mel^{Bal}; dark blue) disruptions. The mating proportions were compared by logistic

regression as above. *sim/mel*^{Dis} females that have a significant reduction in mating have *P*-values shown in bold. D.f.=1.

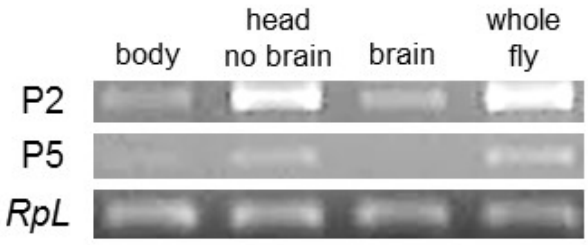

Figure 2-6 Detection of *fruitless* P2 and P5 transcripts in different tissues. RT-PCR product from tissues taken from a female body (thorax and abdomen), head without the brain, brain, and whole fly for *fru* P2, *fru* P5, and the control gene *RpL32*.

To assess whether the *fru* gene is involved in a female's assessment of an individual male courtship component, rather than in the integration of male courtship signals I removed or altered two primary individual components of either male courtship or female perception in mating assays involving female hybrids bearing a *fru* disruption that unmasks the *D. simulans* allele of *fru*. If *fru* is acting via only one of these sensory modalities, then removing that component will increase the receptivity of these hybrids towards *D. melanogaster* males. Removal of the wings of *D. melanogaster* males caused either no effect or a significant reduction in mating when these males were paired with hybrid females bearing a *fru* disruption (*fru*$^{4\text{-}40}$, *fru*^{GAL4}, or *fru*^{MI05459}; Figure 2-7A, Table 2-6).

I then tested whether perception of auditory or olfactory cues in females affects receptivity in the context of *fru* by removing the last two antennal segments and aristae of the female; these are the primary organs for sensing odorants and auditory signals, respectively (Vosshall and Stocker, 2007). I observed no significant difference in copulation upon removal of these organs (Figure 2-7B; Table 2-7). Lastly, I tested whether *fru* is acting via a recently-discovered sensory modality: substrate-borne abdominal vibrations during courtship (Fabre et al., 2012; Mazzoni et al., 2013). I utilized a granite surface to eliminate the transfer of these cues (Elias et al., 2004), but female receptivity did not increase upon removal of substrate vibrations in the context of *fru* (Figure 2-7C; Table 2-8).

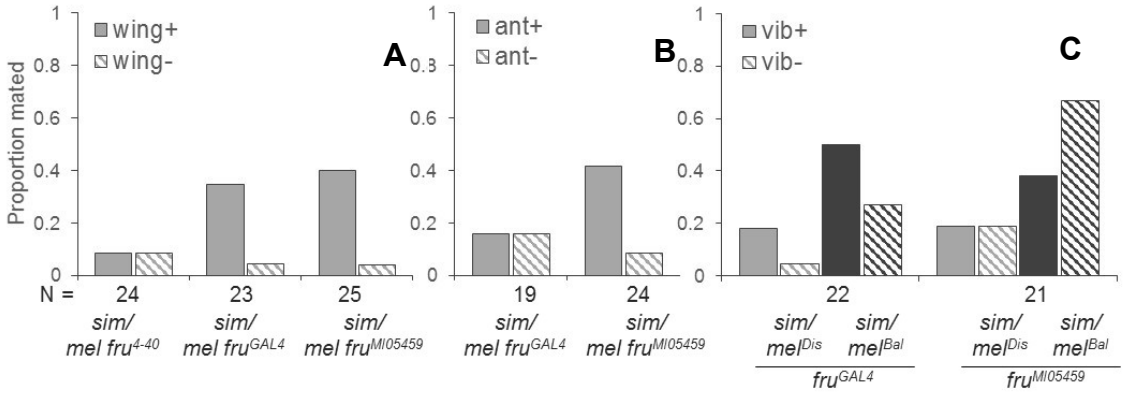

Figure 2-7 Behavioural assays to determine if *fruitless* is acting via an individual sensory modality. (A) Proportion of matings in hybrid females with a *fru disruption* (*sim/mel*^{Dis}) when paired with *D. melanogaster* males that have had their wings removed (wing-) *vs.* those with wings intact (wing+); **(B)** Proportion of matings in hybrid females bearing a *fru* disruption (*sim/mel*^{Dis}) when the female's last two antennal segments have been removed (ant-), which removes the primary sensory organs for both olfactory and auditory cues, compared to females with intact antennae (ant+); or (C) Proportion of matings in hybrid females with a *fru* disruption (*sim/mel*^{Dis}) when the females are placed on a vibrationless (vib-) *vs.* control (vib+) substrate.

Table 2-6 Behavioural assays using hybrid females paired with *D. melanogaster* males with or without wings. Copulation occurrence of hybrid *sim/mel*^{Dis} females when paired in a no-choice mating assay with unaltered (wing+) or wingless (wing-) *D. melanogaster* males. "Time" is the duration of the assay.

D. melanogaster Dis/Bal **genotype used to make hybrid females**	**N**	**wing+ 24h Cop**	**wing- 24h Cop**	**Time**	**Z-value**	***P*-value**
w**; Df(3R)fru*$^{4\text{-}40}$ */ TM3, P[actin-GFP] Ser*^{1} *Sb*^{1}	24	2	2	24h	0.000	1.0000
w**; TI{GAL4}fru*$^{GAL4.P1.D}$ */ TM3, Sb*^{1}	23	8	1	24h	2.602	0.0163
y^{1} *w***; Mi{MIC}fru*^{MI05459} */ TM3, Sb*^{1} *Ser*^{1}	25	10	1	48h	3.073	0.0052

Table 2-7 Behavioural assays using hybrid females with or witouth antennae paired with *D. melanogaster* males. Copulation occurrence of hybrid *sim/mel*^{Dis} females that are intact (ant+) or have their last two antennal segments and aristae removed (ant-), when paired in a 24 hour no-choice mating assay with *D. melanogaster* males.

D. melanogaster Dis/Bal **genotype used to make hybrids**	**N**	**ant+ 24h Cop**	**ant- 24h Cop**	**Z-value**	***P*-value**
y^{1} *w***; Mi{MIC}fru*^{MI05459} */ TM3, Sb*^{1} *Ser*^{1}	24	10	2	2.667	0.007
w**; TI{GAL4}fru*$^{GAL4.P1.D}$ */ TM3, Sb*^{1}	19	3	3	0.000	1.0000

Table 2-8 Behavioural assays using hybrid females paired with *D. melanogaster* males in the presence or absence of substrate vibrations. Copulation occurrence of hybrid females that are placed on media that transmits vibration (vib+) or a granite surface that prevents transmission of substrate vibrations related to courtship (vib-), when paired in a 24 hour no-choice mating assay with D. melanogaster males. Data analyzed by logistic regression; the interaction term of species (hybrid vs. mel) x genotype (Dis vs. Bal) P-value is shown. D.f. = 1.

		vib+		vib-		
		sim/mel ^{Dis}	*sim/mel* ^{Bal}	*sim/mel* ^{Dis}	*sim/mel* ^{Bal}	
***D. melanogaster Dis/Bal* genotype used to make hybrid females**	**N**	**24h Cop**	**24h Cop**	**24h Cop**	**24h Cop**	***P*-value**
y ^{1} *w* **; Mi{MIC}fru* ^{MI05459} */ TM3, Sb* ^{1} *Ser* ^{1}	21	4	8	4	14	0.2461
w **; TI{GAL4}fru* $^{GAL4.P1.D}$ */ TM3, Sb* ^{1}	22	4	11	1	6	0.6657

2.4 Discussion

The results of the initial behavioural assays with *fru* disruptions indicate that hybrid females bearing a *fru* disruption reject *D. melanogaster* males and have a significantly lower mating proportion compared to the controls. A key aspect of this assay is that it is not testing the effect of disrupting a gene's function, since the non-disrupted *D. simulans* allele is functional. Instead, it is assessing naturally-occurring variation in the *D. simulans* allele that is unmasked (Pasyukova et al., 2000). These results confirm that *fruitless* is influencing female receptivity to *D. melanogaster* males and this effect is not due to hemizygosity, as *fru* hemizygous pure-species *D. melanogaster* females did not display a reduction in receptivity. This loss of receptivity is not observed when hybrids with *fru* disruptions are paired with *D. simulans* males. This established that the behaviour observed is not simply a general reduction in female receptivity, but is instead a species-specific rejection in response to *D. melanogaster* males. Further, this reduction in receptivity is not due to defective *fru* processing causing masculinization of mel^{Dis} females as this would be expected to reduce receptivity (Rideout et al., 2007; Aranha et al., 2017), which I did not observe in my control crosses; it also would induce aggression (Manoli et al., 2005; Chan and Kravitz, 2007), which I did not observe in any assays.

The RT-PCR results on the $fru^{MI05459}$ flies indicate that the insertion is affecting the expression of several *fru* transcripts. Although the P1 transcript is detected in $fru^{MI05459}$, $fru^{MI05459}/fru^{4-40}$ males exhibit behavioural sterility as would be expected if the P1 transcript is functionally disrupted (Goodwin et al., 2000; Anand et al., 2001). Flies homozygous for $fru^{MI05459}$ do express P3 and P4 transcripts but do not survive past the pupal stage (Data not shown). However, flies heterozygous for $fru^{MI05459}$ and fru^{sat15} insertions are viable. The fru^{sat15} mutation is derived from the excision of a *P* element inserted into the *fru* gene (Ito et al., 1996), results in a loss of P3 and P4 transcripts and causes lethality in homozygotes (Anand et al., 2001). Since the $fru^{MI05459}/fru^{sat15}$ heterozygous flies show complementation this disruption does not appear to affect the functionality of P3 and P4 transcripts. Behavioural assays with the hybrid females bearing the excised $fru^{MI05459}$ *Minos* element, showed an increase in mating proportion with *D. melanogaster* males for these hybrids. Thus, by removing the disruption in *fru*, I

was able to rescue female receptivity towards *D. melanogaster* males, confirming the effect of *fru* on species-specific female preference.

The precise deletions of *fru* P1 and P2 exons allowed me to test whether transcripts from either of these exons affect female receptivity towards interspecies males. The receptivity of females was tested in two ways. First, for a comparison to the genetic background in which the deletions were made (Canton S), hybrid females with the P1 and P2 disruptions were compared to hybrids made from wild-type Canton S *melanogaster*. Second, to determine the effect of the P1 and P2 deletions on species-specific female preference, I used the same genetic mapping approach as my original assays with the *fru* disruptions. The results of these behavioural assays revealed that hybrid females bearing the P1 or P2 deletions were not less attractive to *D. melanogaster* males and were courted at similar levels to wild-type hybrids females. Additionally, only hybrids females bearing the P2 deletion showed a significant reduction in receptivity to *D. melanogaster* males, but a similar reduction in receptivity was not observed for the P1 deletion. Finally, *D. melanogaster* females homozygous for the P2 deletion were less receptive to *D. melanogaster* males compared to females with the P1 deletion or wild-type Canton S females, but all three groups of females were equally unreceptive to *D. simulans* males. Taken together these results suggest that the P2 transcripts likely have a role in species-specific female mate preference and that the loss of *D. melanogaster fru* P2 significantly reduces female receptivity within species, but the loss of either *fru* P1 or *fru* P2 does not appear to increase female receptivity towards interspecific males. This is the first indication that one of the sex-nonspecific *fru* transcripts may play an important role in behaviour. This finding challenges many theoretical models that consider female preference genes to be separate from genes influencing male traits (e.g., Ritchie et al., 1999; Tomaru et al., 2000). The separate *fru* transcripts encoding female preference and male courtship traits may have facilitated this gene's preference-trait pleiotropy.

The removal of male courtship song did not rescue female receptivity to interspecific males. I used three different *fru* disruptions to test the effect of removing *D. melanogaster* male courtship song on hybrid female receptivity. For all three *fru* disruptions there was no increase in hybrid female receptivity to *D. melanogaster* males,

in fact, for hybrid females bearing the fru^{GAL4} or the $fru^{MI05459}$, there was actually a decrease in mating proportion when they were paired with wingless *D. melanogaster* males. This is similar to what is observed when wings are removed in pure species matings of *D. melanogaster* (Ewing and Bennet-Clark, 1968; Tomaru et al., 2000). Therefore, *fruitless'* effect on species-specific female preference does not appear to be acting via the male courtship song.

Removal of the antennal segments and aristae of hybrid females with *fru* disruptions, did not rescue female receptivity to *D. melanogaster* males. For hybrids bearing the fru^{GAL4} disruption, there was no difference in mating proportion between hybrid females with or without the antennae. For the $fru^{MI05459}$ disruption, antennae-less hybrid females mated significantly less than females with their antennae intact. Since the antennae and the aristae are used for auditory and olfactory perception (Göpfert and Robert, 2002; Gaudry et al., 2012), disrupting the female's ability to sense these cues would rescue female receptivity to interspecific males if *fruitless* is influencing female preference via their perception of auditory and olfactory cues.

Substrate-borne vibrations, are an important component of *Drosophila* male courtship that is often overlooked, and have been shown to affect female receptivity as a response to these male abdominal vibrations (Fabre et al., 2012). Additionally, neurons expressing *fruitless* have been shown to control male abdominal quivering which produces substrate-borne vibrations (Fabre et al., 2012). Therefore, it is possible that *fruitless* might be influencing species-specific female preference through these substrate-borne vibrations, and removal of this courtship component might increase the receptivity of hybrid females with *fru* disruptions to interspecific males. However, the results of the behavioural assays without substrate vibrations showed no such increase in female receptivity in hybrids with *fru* disruptions. Thus, *fru* does not appear to be acting through these substrate vibrations to influence species-specific female preference.

While identifying a single sensory modality through which *fru* is acting to influence female preference would have been an exciting discovery that could help to understand the mechanism by which *fruitless* might be affecting such a complex trait, the answer is

not that simple, and it is likely that *fruitless* might be acting via the integration of sensory inputs from multiple modalities, to affect the females' decision to reject or mate with males. While it is possible that it might be acting through a single cue or sensory organ other than the ones I assessed here, simply removal of the aversive cue from interspecific males might not be sufficient to rescue the receptivity, so future experiments can be designed to test the effect of removing combinations of modalities and replacing the *D. melanogaster* courtship cues with the ones from *D. simulans* males, which might elicit a more substantial preference response from the females.

2.5 Conclusion

Identifying a genetic basis for behavioural isolation is critical for understanding the speciation process and the evolutionary history of such a reproductive barrier (Coyne and Orr, 2004). Here, I identify *fru* as a gene that influences *D. simulans* female rejection of *D. melanogaster* males. This effect appears to be through the P2 group of transcripts of *fru*, which also affects female receptivity within species. This is the first identified behavioural role of a sex-nonspecific transcript of this gene and contradicts established 'good genes' models that predict female preference and male traits are determined by separate loci. The *fru* gene plays a role in sex determination and has highly conserved genetic sequence across the orders Diptera (Davis et al., 2000), Hymenoptera (Bertossa et al., 2009), and Blattaria (Clynen et al., 2011), suggesting an ancient origin and a common function among different insects (Gempe and Beye, 2011). This in addition to its conserved role as a regulator of sexual behaviour warrants further research into characterizing the mechanism by which *fruitless* is influencing female receptivity and behavioural isolation between species.

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Chapter 3

3 Isoforms of the *fruitless* gene influence species-specific female rejection

3.1 Introduction

Reproductive barriers prevent successful mating between individuals from different species (Mayr, 1942). Behavioural isolation is one of the earliest acting and strongest reproductive barriers to hybridization and gene flow between species (Coyne and Orr, 1989, 2004). Typically, behavioural isolation involves incompatibilities in reproductive strategies that result in a lack of sexual attraction between heterospecifics (Coyne and Orr, 2004). For example, in *Drosophila*, males court interspecific females indiscriminately but fail to elicit receptivity from these females due inappropriate sensory cues (Ting et al., 2001; Coyne and Orr, 2004). As with many insect species, copulation success in *Drosophila* ultimately depends on female willingness to mate (Greenspan, 1995). Female preference can therefore contribute to the formation and maintenance of reproductive barriers. Therefore, identifying genetic variants that influence species-specific female mate preference can enhance our understanding of the genetic mechanisms of reproductive barriers, how these barriers evolve and how they contribute to the isolation of species. To date, a small number of genomic regions influencing variation in species-specific female preference have been identified (Campesan et al., 2001; Fitzpatrick et al., 2005; Kronforst et al., 2006; Moehring et al., 2006; Laturney and Moehring, 2012a), but no individual gene affecting this trait has been confirmed.

Drosophila has been the subject of research into the factors that promote behavioural isolation (Coyne and Orr, 1989; Nanda and Singh, 2012). Within the genus, the *D. melanogaster* and *D. simulans* species pair is of interest because they are behaviourally isolated (Carracedo et al., 2000) and are amenable to genetic and molecular analysis. Specifically, genomic tools allow for the detailed characterization of genetic and neural basis for complex behaviours. *Drosophila melanogaster* and *D. simulans* are asymmetrically isolated from one another, meaning female *D. simulans* reject *D. melanogaster* males (Sturtevant, 1920; Barker, 1967; Carracedo et al., 2000) but *D.*

melanogaster females mate readily with *D. simulans* males. This permissive cross produces sterile hybrid females that are receptive to *D. melanogaster* males (Sturtevant, 1920; Manning, 1959), whereas the non-permissive cross do not produce any hybrids. This pattern indicates that loci contributing to *D. simulans* rejection of *D. melanogaster* males are effectively recessive. As traditional recombination mapping techniques are not possible between these two species due to a lack of their ability to produce fertile hybrid offspring, an alternative mapping approach such as deficiency mapping can be used to identify genomic regions and individual genes for the recessive *D. simulans* rejection behaviour. A previous study used deficiency mapping to identify several regions in the right arm of the 3rd chromosome that contain potential candidate genes (Laturney & Moehring, 2012b). One such region was further mapped using smaller deletions within these regions to identify *fruitless* (See Chapter 2). Subsequent assays using *fruitless* disruptions $fru^{4\text{-}40}$, fru^{NP0021} and fru^{GAL4} (Lee et al., 2000; Anand et al., 2001; Stockinger et al., 2005; Kimura et al., 2005, 2008) showed that when the *D. melanogaster* allele of *fruitless* in an interspecies hybrid is disrupted (so that only the *D. simulans* allele is functional) female receptivity towards *D. melanogaster* males is reduced compared to controls. I predict therefore that *fruitless* is affecting species-specific female preference through neural circuitry that controls the female's processing and evaluation of interspecific mating signals.

The *fruitless* gene has several key functions in *Drosophila* - namely, to regulate sex determination and development of male courtship behaviour (Ryner et al., 1996). The importance of *fruitless* extends beyond male courtship and sex determination, and it is shown to be necessary for external morphology such as the formation of the male specific "Muscle of Lawrence" (MOL) (Lawrence and Johnston, 1984; Gailey et al., 1991), which is required for the male's ability to curl its abdomen during copulation attempts (Usui-Aoki et al., 2000). It is also essential for viability in both sexes (Ryner et al., 1996; Anand et al., 2001).

fruitless undergoes alternative splicing at the 5' and 3' ends to produce putative transcripts initiating with one of five first exons (P1-P5), These exons all contain a common BTB (broad complex, tramtrack, bric-a-brac) domain, and one of four possible

3’ exons (A-D) which contain a zinc finger domain (Ito et al., 1996; Ryner et al., 1996). The center of each transcript contains the same set of five exons, called the ‘common’ domains (C1-5). *fruitless* transcripts from the most distal promoter P1 are sex-specifically spliced to produce male and female transcripts. The male-specific P1 transcripts are translated into Fru^M proteins while the female P1 transcripts, although detectable in the central nervous system of females, are not translated into proteins (Ito et al., 1996; Ryner et al., 1996; Usui-Aoki et al., 2000). Fru^M proteins are expressed in approximately 3% of the neurons in the central nervous system as well as the neurons innervating the abdominal ganglion and genitalia (Billeter and Goodwin, 2004). Fru^M is first detected during the early pupal stages when the central nervous system is remodelled (Billeter et al., 2006; Lee et al., 2000; Neville et al., 2014).

The majority of research on *fruitless* has been centered on P1 transcripts, but *fruitless* is a pleiotropic gene with additional roles beyond those of P1 in male courtship and sex determination (Billeter et al., 2006; Song et al., 2002). A subset of the non sex-specifically spliced transcripts (P2-P5) are necessary for adult viability and external morphology (Song et al., 2002). The P2 transcripts are strongly expressed in the eye (Leader et al., 2017). The P3 and P4 transcripts are, by contrast, primarily expressed in the developing CNS, and may be involved in the formation of axonal tracts (Song et al., 2002). The P1 transcripts do not appear to affect female mating behaviour within species (Baker et al., 2001), while transcripts starting with the P2 exon affect species-specific female preference.

Each of the *fru* transcripts ends with one of four possible 3’ exons. As such, there can be four alternative protein products (Fru^A, Fru^B, Fru^C, and Fru^D) from each different 5’ exon (P1-P5). These 3’ exons encode the C-terminal zinc-finger (Zn-finger) DNA-binding domains of *fru* proteins. The male specific P1 transcripts produce three different Fru^M protein isoforms with alternative C-terminal Zinc-finger domains (Billeter et al., 2006). A previous study by Neville et al., (2014) generated *fru* isoform-specific mutants with premature stop codons inserted in each individual 3’ exon ($fru^{\Delta A}$, $fru^{\Delta B}$, and $fru^{\Delta C}$) that are unable to produce any of the full length *fru* isoforms with A, B or C exons. Analysis of these mutants revealed that Fru^B and Fru^C isoforms are essential for male courtship and

development, while Fru^A isoforms seem to have a more nuanced effect on mating behaviour (Neville et al., 2014).

In this chapter, I assessed whether the individual Fru^A, Fru^B, and Fru^C isoforms affect species-specific female preference through a genetic mapping approach using the *fru* isoform specific mutants $fru^{\Delta A}$, $fru^{\Delta B}$, or $fru^{\Delta C}$. I also investigate whether female receptivity towards interspecific males can be rescued by expressing the individual *fru* isoforms in *fru* mutant hybrids using the *GAL4/UAS* system. I use *UAS-fru* transgenes containing only the common region (C1-5) coding sequence and ending with one of the 3' *fru* exons (A-C). These *UAS-fru* transgenes should encode *fru* proteins similar to those from the non-sex specific transcripts of *fruitless*, i.e. P2, P3 and P4 (Song et al., 2002). I use two different *GAL4* drivers (fru^{GAL4} and fru^{NP0021}) to express the *UAS-fru* transgenes in a *fru*-specific pattern in hybrid females to determine if they rescue species-specific female preference. This will provide additional evidence in support of *fruitless* as a regulator of species-specific female preference. Further, if any or all *fru* isoforms are involved in species-specific female preference, it can help identify potential downstream genes and also characterize neural circuitry governing female preference.

3.2 Methods

***Drosophila* Strains and Crosses:** Flies were maintained on standard food medium (Bloomington *Drosophila* Stock Center) under a 14:10 light:dark cycle at 24ºC and approximately 80% relative humidity. The fru^{NP0021} stock was obtained from the Bloomington *Drosophila* Stock Center (Bloomington, Indiana) as was stock #3703 ($w^{1118}/Dp(1;Y)y^+$; CyO/nub^1 b^1 sna^{Sco} lt^1 stw^3; $MKRS/TM6B$, Tb^1). The fru^{GAL4} (Stockinger et al., 2005) and the *UAS-fru* lines generated by random insertion using *P*-element transgenesis (Song et al., 2002) were provided by Dr. Barbara Taylor. The $fru^{\Delta A}$, $fru^{\Delta B}$, and $fru^{\Delta C}$ disruption lines (Neville et al. 2014) and the *UAS-fru* lines generated by site-specific insertion were provided by Dr. Stephen Goodwin. For this latter set of stocks, I maintained disruptions over the 3rd chromosome balancer *TM3* that contained a phenotypic marker to identify recombinant offspring. The *D. melanogaster* stock with GFP-tagged sperm (*w; P{w8, ProtA-EGFP, w+}19B(3)*) was provided by Dr. John Belote. Wild-type *D. melanogaster* BJS is an isofemale line collected by Dr. Brent

Sinclair. Wild-type *D. simulans* Florida City (FC) was provided by Dr. Jerry Coyne (collected from Florida City, Florida; (Coyne, 1989).

Testing the effect of the *fru* isoform specific mutations in species-specific female rejection: Five to seven virgin females from each of the $fru^{\Delta A}$, $fru^{\Delta B}$, and $fru^{\Delta C}$ disruptions (*Dis*) balanced over *TM3* (*Bal*) were aged 5 days and crossed with 25-30 *D. simulans* FC males or 4-5 *D. melanogaster* males to produce hybrid sim/mel^{Dis} and sim/mel^{Bal} or mel/mel^{Dis} and mel/mel^{Bal} offspring, respectively. F1 hybrid females of these four genotypes were aged 5- 7 days and paired with age-matched virgin *D. melanogaster* males. New sentence... with GFP-tagged sperm, in no-choice mating assays. Assays were carried out at 24ºC, ~50% relative humidity, and 1-2 hours of "lights on" in 30mL plastic vials containing approximately 5 mL of standard food medium. All four genotypes were assayed in the same day in equal numbers to account for environmental effects that could influence mating activity (Austin, et al. 2014). The assays were observed for 1 hour and courtship latency, duration and the proportion of copulation out of those that were courted was calculated. The F1 hybrid sim/mel^{Dis} and sim/mel^{Bal} females were left in the food vials for 24 hours, after which point the females were decapitated and their reproductive tracts were dissected. The presence of fluorescently labelled sperm was used to score for copulation occurrence. I used logistic regression ($\alpha < 0.05$) to compare the proportion of females that copulated out of those that were courted where species (*mel/mel* or *mel/sim*) and genotype (*Dis* or *Bal*) were the independent variables, and copulation occurrence after courtship (*yes or no*) was the dependent variable. The logistic regression compared the likelihood ratios between expected and observed counts for each category. The species × genotype interaction term was the effect of interest and a result was considered to be biologically significant only if the sim/mel^{Dis} females had reduced copulation compared to sim/mel^{Bal} (interspecies control), after the values are corrected for any effects of the balancer or disruption chromosomes themselves, as determined using mel/mel^{Dis} and mel/mel^{Bal} (intraspecies controls).

Transgenic *GAL4-UAS* mediated expression of *fru* isoforms: To test whether species-specific female rejection can be rescued by expressing specific *fru* isoforms using the *GAL4-UAS* expression system, I tested two sets of *UAS-fru* transgenes with the common

region (C1-5) coding sequence and one of the 3' *fru* exons (A-C). Each one of the three isoforms were cloned separately into a *UAS* vector and inserted into the second chromosome (Song et al., 2002). Individual *UAS-fru* transgene constructs were randomly inserted into the second chromosome via *P*-element transgenesis (labelled "R": *UAS-fru*$^{A\text{-}R}$, *UAS-fru*$^{B\text{-}R}$, and *UAS-fru*$^{C\text{-}R}$). Separately, one set of the three *UAS-fru* transgenes were individually inserted into an attP site on the second chromosome (labelled "T": *UAS-fru*$^{A\text{-}T}$, *UAS-fru*$^{B\text{-}T}$, and *UAS-fru*$^{C\text{-}T}$) (Goodwin unpublished). I used two different *GAL4* drivers (*fru*^{GAL4} and *fru*^{NP0021}) that were used to express *UAS-fru* transgenes in a *fru*-specific manner, via their insertion in *fru* and simultaneously disrupts sex-specific *fru* expression resulting in male courtship and fertility defects (Stockinger et al., 2005).

5-7 virgin females from *fru*^{GAL4} and *fru*^{NP0021} were crossed with 1-3 *y*^{1} *w***; CyO/nub*^{1} *b*^{1} *sna*^{Sco} *lt*^{1} *stw*^{3}*; MKRS/TM6B, Tb*^{1} males to generate flies of the following genotypes: *y*^{1} *w***; CyO/nub*^{1} *b*^{1} *sna*^{Sco} *lt*^{1} *stw*^{3}*; fru*^{GAL4}*/TM6B, Tb*^{1} and *y*^{1} *w***; CyO/nub*^{1} *b*^{1} *sna*^{Sco} *lt*^{1} *stw*^{3}*; fru*^{NP0021}*/TM6B, Tb*^{1}. Males from either of these stocks were crossed with *yw; UAS-fru; MKRS/TM6B, Tb*^{1} females to produce females bearing *GAL4* and *UAS* constructs (*yw; UAS-fru/CyO ; GAL4/MKRS*). Five to seven virgin females of these genotypes were crossed with 25-30 *D. simulans* FC males to produce hybrids with both *GAL4* and *UAS-fru* constructs (*GAL4-UAS/sim*). Concurrently, 5-7 virgin females *fru*^{GAL4} or *fru*^{NP0021} females were crossed with 25-30 *D. simulans* males to produce *GAL4* hybrids (*GAL4/sim*). These would act as negative controls, since both *fru*^{GAL4} and *fru*^{NP0021} are known disruptions of *fruitless* (Kimura et al., 2005; Stockinger et al., 2005) and also affect species-specific female preference (see Chapter 2). In addition to these, 5-7 virgin females with the genotype *yw; UAS-fru/CyO; MKRS/TM6B, Tb*^{1} were crossed with *D. simulans* FC males to produce *UAS-fru* hybrids (*UAS Bal/sim*). Approximately 30 vials of crosses were set up to generate hybrid females of the three genotypes.

F1 hybrid females with the three aforementioned genotypes were then aged 5-7 days and paired with 5-7 day old virgin *D. melanogaster* males with GFP-tagged sperm. Copulation success was scored in a 24 hour no choice mating assay, as above, and the proportions copulated for each genotype were compared using Z-tests ($\alpha < 0.05$).

3.3 Results

I found that female hybrids with $fru^{\Delta A}$, $fru^{\Delta B}$, or $fru^{\Delta C}$ mutations reduce their mating receptivity towards *Drosophila melanogaster* males (Figure 3-1; Table 3-1). This rejection is species-specific (hybrid females with complete loss of *D. melanogaster fru* are still receptive towards *D. simulans* males; (See Chapter 2)). This pattern suggests that loss of any one of the Fru^{A}, Fru^{B} or Fru^{C} isoform groups is sufficient to induce *D. simulans*-like female rejection.

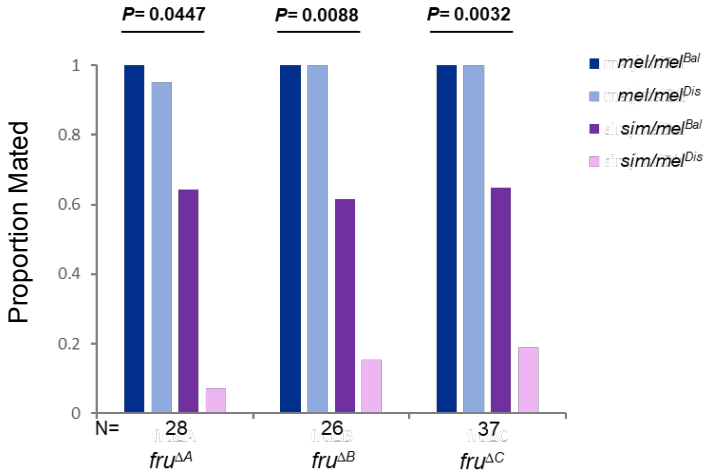

Figure 3-1 Behavioural assays with *fruitless* isoform mutants paired with *D. melanogaster* males. Proportion of females mated when paired with *D. melanogaster* males for control pure species females without a disruption (mel/mel^{Bal}), with a disruption (mel/mel^{Dis}), and hybrid females without a disruption (sim/mel^{Bal}), when compared to hybrid females with a disruption (sim/mel^{Dis}). Comparisons where sim/mel^{Dis} females have a significant reduction in mating have *P*-values shown in bold. The proportions of matings were compared using logistic regression. Results were considered biologically significant only if the sim/mel^{Dis} females had reduced copulation compared to sim/mel^{Bal}, after values are corrected for effects of the balancer or disruption, determined by the mel/mel^{Dis} and mel/mel^{Bal} controls.

Table 3-1 Behavioural assays with hybrids bearing *fru*$^{\Delta A}$, *fru*$^{\Delta B}$, and *fru*$^{\Delta C}$ disruption lines. Females were paired with *D. melanogaster* males. The number of courtships that occurred within 1 hour (Court) as well as the number of copulations in 1 hour (1h Cop) were recorded; hybrid females were also assessed for copulation occurrence after 24 hours (24h), after all females were presumed to have experienced courtship. Data analyzed by logistic regression; the interaction term of species (hybrid vs. mel) x genotype (Dis vs. Bal) determines if a result is biologically significant. P-value is shown. D.f. = 1.

				sim/mel^{Bal}			*sim/mel*^{Dis}			*mel/mel*^{Bal}		*mel/mel*^{Dis}		
Name	***D. melanogaster* Dis/Bal genotype used to make hybrid females**	**Replicate**	**N**	**Court**	**1h Cop**	**24h Cop**	**Court**	**1h Cop**	**24h Cop**	**Court**	**1h Cop**	**Court**	**1h Cop**	***P*-value**
fruΔA	*Fru A (Deletion) / TM3, Sb*^{1} *Ser*^{1}	1	28	23	9	18	22	2	6	21	21	21	20	0.04471
fruΔB	*Fru B (Deletion) / TM3, Sb*^{1} *Ser*^{1}	1	26	24	10	16	23	0	4	19	19	20	20	0.00884
fruΔC	*Fru C (Deletion) / TM3, Sb*^{1} *Ser*^{1}	1	37	34	8	24	34	2	7	27	27	29	29	0.00322

Table 3-2 Behavioural assays with hybrid females bearing targeted and randomly inserted *UAS-fru*^{A} constructs paired with *fru*^{GAL4} or *fru*^{NP0021} *GAL4* drivers. Females were paired with *D. melanogaster* males. The number of courtships that occurred within 1 hour (Court) as well as the number of copulations in 1 hour (1h Cop) were recorded; hybrid females were also assessed for copulation occurrence after 24 hours (24h), after all females were presumed to have experienced courtship. Mating proportions of *sim/mel*$^{GAL4+UAS}$, were compared to positive controls (*sim/mel*^{GAL4}) and negative controls (*sim/mel*$^{UAS\ Bal}$). Data was analyzed by *Z*-test ($\alpha < 0.05$).

				sim/mel$^{GAL4+UAS}$			*sim/mel*^{GAL4}			*sim/mel*$^{UAS\ BAL}$			GAL4+UAS vs GAL4		GAL4+UAS vs UAS BAL	
Name	***D. melanogaster* genotype used to make hybrid females**	**GAL4 driver**	**N**	**Court**	**1h Cop**	**24h Cop**	**Court**	**1h Cop**	**24h Cop**	**Court**	**1h Cop**	**24h Cop**	**Z**	***P*-value**	**Z**	***P*-value**
UAS-fru^{A-R} *(Random insertion)*	*w; UAS fruA/CyO; fru*^{GAL4}*/MKRS*	*fru*^{GAL4}	43	36	0	6	36	3	8	35	4	17	0.5842	0.56192	2.6798	0.00736
UAS-fru^{A-T} *(Targeted insertion)*	*w; UAS fruA/CyO; fru*^{GAL4}*/MKRS*	*fru*^{GAL4}	21	17	0	3	17	2	7	16	2	9	1.4491	0.07353	2.0494	0.02018
UAS-fru^{A-T}*(Targeted insertion)*	*w; UAS fruA/CyO; fru*^{NP0021}*/MKRS*	*fru*^{NP0021}	34	29	0	11	29	3	12	32	9	24	0.2563	0.79486	3.1543	0.00164

Based on the results of the *GAL4/UAS* rescue experiments, expression of the Fru^{A} isoform does not restore female receptivity. Female hybrids with either the fru^{GAL4} or fru^{NP0021} *GAL4* paired with either *UAS-*fru^{A-R} or fru^{A-T} constructs did not have increased receptivity towards *D. melanogaster* males (Figure 3-2; Table 3-2). Instead, they showed significantly lower mating proportions compared to positive control females (hybrids with the *UAS-*fru^{A} only) and comparable mating proportions to the negative control females (i.e., hybrids with the *GAL4* only).

Likewise, Fru^{B} does not rescue female receptivity. Female hybrids with fru^{GAL4} or fru^{NP0021} *GAL4* paired with *UAS-*fru^{B-T} did not have increased receptivity towards *D. melanogaster* males (Figure 3-3; Table 3-3). As with *UAS-*fru^{A}, there was lower mating proportions of fru^{GAL4}; *UAS-*fru^{B-T} females compared to positive control females and comparable mating proportions to the negative control females. There was, however, a strong decrease in receptivity with fru^{NP0021}*GAL4*; *UAS-*fru^{B-T}, where females showed significantly reduced mating compared to both positive and negative controls (Figure 3-3A, B; Table 3-3). Because of the low proportion of matings observed in fru^{NP0021}GAL4; *UAS-*fru^{B-T} hybrid females, I tested whether there was a general reduction in female receptivity caused by expression of these site-specific *UAS-*fru^{B-T} transgenes, or if it is a hybrid-specific effect. I found that expression of the site-specific transgene does not reduce female receptivity compared to controls (Table 3-4).

I was unable to test the randomly inserted *UAS-*fru^{B-R} transgenes' effect on hybrid female receptivity as *D. melanogaster* females bearing the *UAS-*fru^{B-R} and fru^{GAL4} constructs did not produce any hybrid offspring with *D. simulans* males. Approximately 50 different vials of crosses were set up to produce these hybrids but unfortunately no larvae were observed for any of these crosses. However, the receptivity of these females towards *D. melanogaster* males was unaffected (Data not shown).

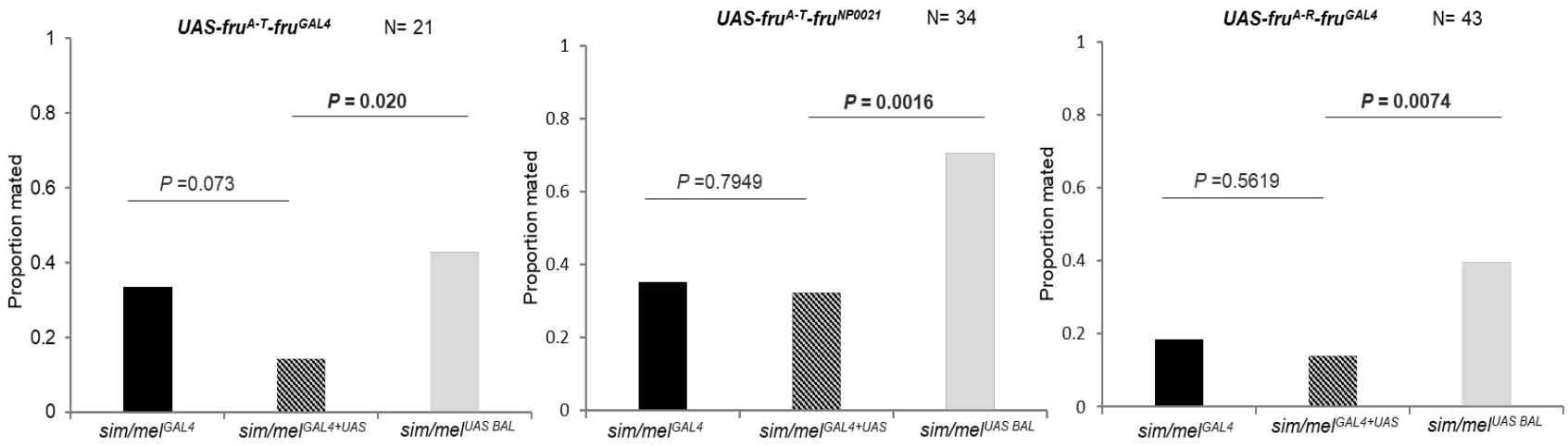

Figure 3-2 Behavioural assays with hybrid females bearing targeted and randomly inserted *UAS-fru*^{A} constructs activated by *fru* specific *GAL4* drivers. Mating Proportion of hybrid females paired with *D. melanogaster* males. Females with both *UAS-fru*^{A} and *GAL4* transgenes (*sim/mel*$^{GAL4+UAS}$) compared to positive control females (*sim/mel*$^{UAS\ BAL}$) and negative control females (*sim/mel*^{GAL4}). Mating proportions were compared using a *Z*- test $\alpha < 0.05$**.** Comparisons where the *sim/mel*$^{GAL4+UAS}$ have a significant difference with the positive or negative controls have *P*-values shown in bold**.**

Table 3-3 Behavioural assays with hybrid females bearing targeted *UAS-fru^{B}* constructs paired with *fru^{GAL4}* or *fru^{NP0021}* drivers. Females were paired with *D. melanogaster* males. The number of courtships that occurred within 1 hour (Court) as well as the number of copulations in 1 hour (1h Cop) were recorded; hybrid females were also assessed for copulation occurrence after 24 hours (24h), after all females were presumed to have experienced courtship. Mating proportions of *sim/mel$^{GAL4+UAS}$*, were compared to positive controls (*sim/mel^{GAL4}*) and negative controls (*sim/mel$^{UAS\ Bal}$*). Data was analyzed by *Z*-test ($\alpha < 0.05$).

				sim/mel $^{GAL4+UAS}$			*sim/mel* ^{GAL4}			*sim/mel* $^{UAS\ BAL}$			GAL4+UAS vs GAL4		GAL4+UAS vs UAS BAL	
Name	*D. melanogaster* genotype used to make hybrid females	GAL4 driver	N	Court	1h Cop	24h Cop	Court	1h Cop	24h Cop	Court	1h Cop	24h Cop	Z	*P*-value	Z	*P*-value
UAS-fruB $^{-T}$ *(Targeted insertion)*	*w; UAS fruB/CyO; fru* ^{GAL4}*/MKRS*	*fru* ^{GAL4}	20	16	0	4	15	1	4	18	4	11	0	0.5	2.2862	0.01101
UAS-fruB $^{-T}$*(Targeted insertion)*	*w; UAS fruB/CyO; fru* ^{NP0021}*/MKRS*	*fru* ^{NP0021}	34	31	0	1	26	4	10	30	2	17	2.9639	0.00154	4.398	0.00001

Table 3-4 Behavioural assays with pure species *D. melanogaster* females bearing site-specific *UAS-fru^{B}* and *fru^{NP0021} GAL4* drivers. Females were paired with *D. melanogaster* GFP sperm males. The number of courtships that occurred within 1 hour (Court) as well as the number of copulations in 1 hour (1h Cop) were recorded. Mating proportions of *mel/mel$^{GAL4+UAS}$*, were compared to positive controls (*mel/mel^{GAL4}*) and negative controls (*mel/mel$^{UAS\ Bal}$*). Data was analyzed by *Z*-test ($\alpha < 0.05$).

				mel/mel $^{GAL4+UAS}$		*mel/mel* ^{GAL4}		*mel/mel* $^{UAS\ BAL}$		GAL4+UAS vs GAL4		GAL4+UAS vs UAS BAL	
Name	*D. melanogaster* genotype used to make GAL4-UAS females	GAL4 driver	N	Court	1h Cop	Court	1h Cop	Court	1h Cop	Z	*P*-value	Z	*P*-value
UAS-fruB $^{-T}$*(Targeted insertion)*	*w; UAS fruB/CyO; fru* ^{NP0021}*/MKRS*	*fru* ^{NP0021}	26	26	22	25	21	26	22	0.3666	0.356	0	0.5

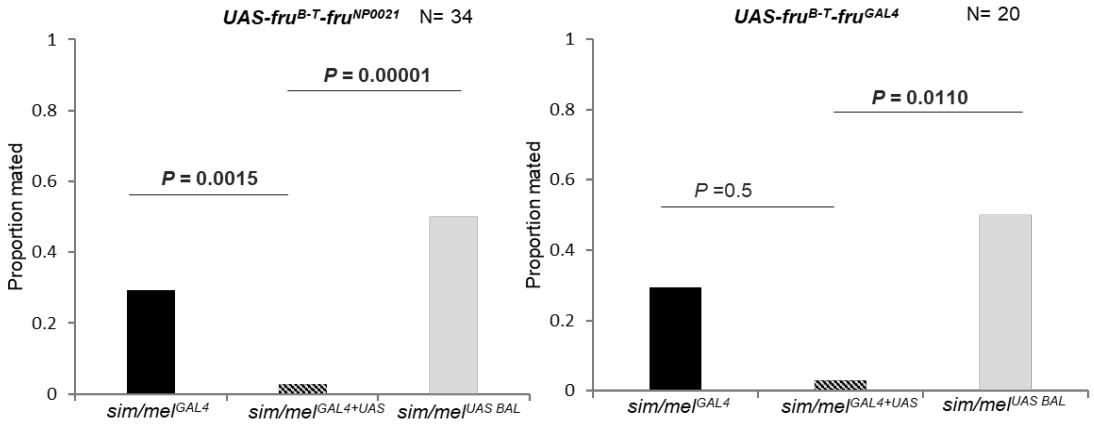

Figure 3-3 Behavioural assays with hybrid females bearing site specific *UAS-fru*^{B} constructs activated by *fru* specific *GAL4* drivers. Proportion of hybrid females that mated when paired with *D. melanogaster* males for females with both *UAS-fru*$^{B-T}$ and *GAL4* transgenes (*sim/mel*$^{GAL4+UAS}$) compared to positive control females (*sim/mel*$^{UAS\ BAL}$) and negative control females (*sim/mel*^{GAL4}). Mating proportions were compared using a *Z*-test $\alpha < 0.05$. Comparisons where the *sim/mel*$^{GAL4+UAS}$ have a significant difference with the positive or negative controls have *P*-values shown in bold.

Only one of the *UAS-fru*^{C} insertions rescued female receptivity in hybrids. Expression of the site specific *UAS-fru*$^{C-T}$ transgene driven by *fru*^{GAL4} or *fru*^{NP0021} failed to restore receptivity to *D. melanogaster* males, as these females showed significantly reduced mating compared to the positive control females, and similar mating proportions compared to the negative controls (Figure 3-4; Table 3-5). However, the *UAS-fru*$^{C-R}$ lines generated by random insertion restored *D. melanogaster*-like receptivity in *fru* mutant hybrids when paired with the *fru*^{GAL4} driver. These females with Fru^{C} expression exhibited significantly higher mating proportions compared to the negative control females (*fru*^{GAL4} only) and comparable mating proportions to the positive control females (*UAS-fru*$^{C-R}$ only) (Figure 3-5; Table 3-5). These results indicate that expression of the Fru^{C} isoforms was sufficient to rescue female receptivity in *fru* mutant hybrids.

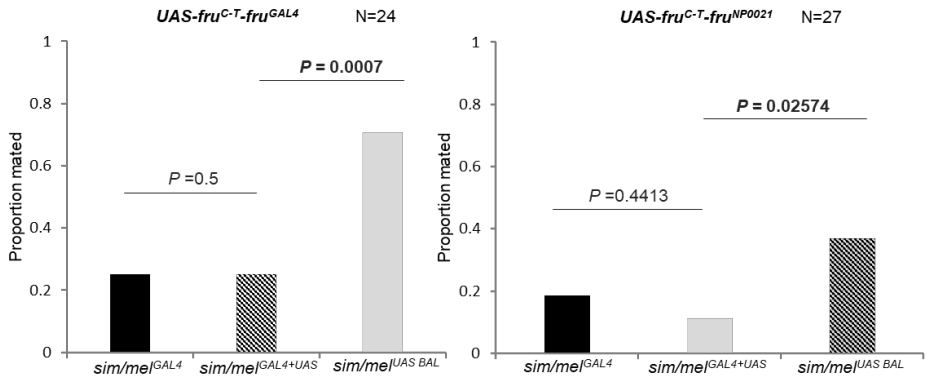

Figure 3-4 Behavioural assays with hybrid females bearing targeted *UAS-fru^{C}* constructs activated by *fru* specific *GAL4* drivers. Proportion of hybrid females that mated when paired with *D. melanogaster* males for females with both *UAS-fru^{C-T}* and *GAL4* transgenes (*sim/mel$^{GAL4+UAS}$*) compared to positive control females (*sim/mel$^{UAS\ BAL}$*) and negative control females (*sim/mel^{GAL4}*). Mating proportions were compared using a *Z*-test. Comparisons where the *sim/mel$^{GAL4+UAS}$* have a significant difference with the positive or negative controls have *P*-values shown in bold.

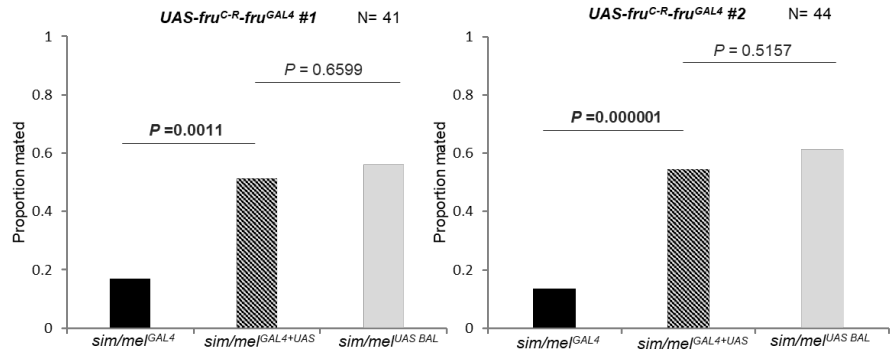

Figure 3-5 Behavioural assays with hybrid females bearing randomly inserted *UAS-fru^{C}* constructs activated by the *fru^{GAL4}* driver. Proportion of hybrid females that mated when paired with *D. melanogaster* males for females with both *UAS-fru^{C-T}* and *GAL4* transgenes (*sim/mel$^{GAL4+UAS}$*) compared to positive control females (*sim/mel$^{UAS\ BAL}$*) and negative control females (*sim/mel^{GAL4}*). Mating proportions were compared using a *Z*-test Comparisons where the *sim/mel$^{GAL4+UAS}$* have a significant difference with the positive or negative controls have *P*-values shown in bold.

Table 3-5 Behavioural assays with female hybrids carrying targeted or randomly inserted *UAS-fru^{C}* constructs activated by and *fru^{GAL4}* or *fru^{NP0021}* *GAL4* drivers. Females were paired with *D. melanogaster* GFP sperm males. The number of courtships that occurred within 1 hour (Court) as well as the number of copulations in 1 hour (1h Cop) were recorded; hybrid females were also assessed for copulation occurrence after 24 hours (24h), after all females were presumed to have experienced courtship. Mating proportions of *sim/mel$^{GAL4+UAS}$*, were compared to positive controls (*sim/mel^{GAL4}*) and negative controls (*sim/mel$^{UAS\ Bal}$*). Data was analyzed using a *Z*-test ($\alpha < 0.05$).

				sim/mel$^{GAL4+UAS}$			*sim/mel*^{GAL4}			*sim/mel*$^{UAS\ BAL}$			GAL4+UAS vs GAL4		GAL4+UAS vs UAS BAL	
Name	***D. melanogaster* genotype used to make hybrid females**	**GAL4 driver**	**N**	**Court**	**1h Cop**	**24h Cop**	**Court**	**1h Cop**	**24h Cop**	**Court**	**1h Cop**	**24h Cop**	**Z**	***P*-value**	**Z**	***P*-value**
UAS-fruC^{-R} (Randoninsertion)#1	*w; UAS fruC/CyO; fru^{GAL4}/MKRS*	*fru^{GAL4}*	41	39	1	21	41	0	7	37	7	23	3.2604	0.00112	0.4428	0.65994
UAS-fruC^{-R} (Randoninsertion)#2	*w; UAS fruC/CyO; fru^{GAL4}/MKRS*	*fru^{GAL4}*	44	28	0	24	30	1	6	32	7	27	4.048	0.000001	0.6479	0.5157
UAS-fruC^{-T} (Targeted insertion)	*w; UAS fruB/CyO; fru^{GAL4}/MKRS*	*fru^{GAL4}*	24	18	0	6	17	2	6	17	3	17	0	0.5	3.1782	0.0007
UAS-fruC^{-T}(Targeted insertion)	*w; UAS fruB/CyO; fru^{NP0021}/MKRS*	*fru^{NP0021}*	27	26	0	3	24	0	5	27	1	10	0.7661	0.4413	2.2281	0.02574

3.4 Discussion

The rescue of female receptivity with *UAS-fru*$^{C-R}$ but not *UAS-fru*$^{C-T}$ suggests that there are position effects that may vary the expression of *UAS-fru* transgenes. These transgenes were identical and only differed in their site of insertion in the genome. Position-effect variegation is known to affect gene expression as a result of changes in the location of the gene (Elgin and Reuter, 2013). One of the earliest identified cases was in the *white* gene which controls eye colour in *Drosophila.* In mutants the *white* gene was silenced in some cells resulting in a variegated eye colour (Muller, 1930). This was a consequence of chromosomal rearrangements resulting in a change in the gene's location that altered its expression (Weiler and Wakimoto, 1995; Elgin and Reuter, 2013). This variation in expression is often attributed to local enhancers and regulatory elements that affect gene expression (O'Kane and Gehring, 1987). In the case of the *UAS-fru*^{C} transgenes, presence of potential regulatory elements in the vicinity of the insertion site may affect their expression considerably, which could explain the difference in the results of the mating assays. Indeed, the potential for positional effects makes it difficult to rule out the potential role that *fru*^{A} or *fru*^{B} could also play in restoring female receptivity, even though both random and targeted insertions of *UAS-fru*^{A} and *UAS-fru*^{B} transgenes failed to restore female receptivity.

Taken together, these results indicate that the individual *fru* isoforms are necessary for the production of *D. melanogaster*-like female receptivity, but expression of only the Fru^{A} or Fru^{B} isoforms is likely not sufficient to rescue *D. melanogaster*-like female receptivity. Only the Fru^{C} isoform was able to restore female receptivity towards *D. melanogaster* males. In addition to potential positional effects, another potential caveat of these rescue experiments is that the drivers used to express the *UAS-fru* transgenes might not recapitulate *fru* expression in the relevant cells necessary to rescue female receptivity. The *fru*^{GAL4} and the *fru*^{NP0021} drive expression in a predominantly *fru* P1 specific pattern that is not similar to *fru* P2 expression (Dornan et al., 2005; Kimura et al., 2005; Stockinger et al., 2005). Based on my findings from Chapter 2, only the *fru* P2 transcripts are involved in species-specific female receptivity, and so the expression of these *UAS-fru* transgenes might be limited to subsets of neurons that are not involved in influencing

species-specific female receptivity. This could also explain why a rescue is only observed with the Fru^C isoform expression, as the fru^{GAL4} driver may induce the relevant expression patterns for Fru^C and not for Fru^A or Fru^B.

The various *fru* isoforms have unique binding specificities and target various genes involved in nervous system development (e.g., *CadN, lola,* and *pdm2*; Nojima, Neville, & Goodwin, 2014). The male-specific Fru^A, Fru^B, and Fru^C isoforms starting with the P1 first exon have distinct roles in male courtship. P1-Fru^C isoforms are necessary for the species-specific inter-pulse intervals in courtship song. Males lacking these isoforms exhibit significantly higher inter-pulse intervals than is typical for *D. melanogaster* males, but are comparable to the inter-pulse intervals observed in *D. simulans* males (Neville et al., 2014). Additionally, $fru^{\Delta C}$ mutants exhibit the most severe courtship defects and fail to initiate copulation due to a lack of the male-specific Muscle of Lawrence (Neville et al., 2014; Von Philipsborn et al., 2014). Fru^C isoforms are also necessary for specifying sexual dimorphism in neural patterns, as loss of Fru^C isoforms in vAB3 or aSP4 neuronal classes results in feminization of arborisation patterns (Von Philipsborn et al., 2014).

Genome-wide identification of *fru* isoform targets showed a considerable overlap in the putative gene targets for the three isoforms, despite the distinctive DNA binding domains of each isoform (Dalton et al., 2013; Vernes, 2014). Roughly 60 regulatory gene targets were shared by all three *fru* isoforms, including genes involved in courtship behaviour, such as *sex peptide receptor* (*SPR*) and *Shaker* (*Sh*) (Vernes, 2014). Many of these putative target genes also share protein domains like the immunoglobulin-like domains and p53/RUNT-type transcription factor DNA-binding domains. However, each isoform also shows differences in the gene target groups. The Fru^C isoform targets contain an enriched level of neurotransmitter receptor activity genes, including *Dopamine 2-like receptor* (*Dop2R*). In contrast, the Fru^A isoform targets show an overrepresentation of genes involved in transmembrane signalling receptor activity, including genes such as *sevenless* (*sev*) and *white* (*w*). The Fru^B target list was considerably enriched for genes involved in neuromuscular junction development and include *Neuroligin 1* (*Nlg1*), *futch* and *cacophany* (*cac*) (Vernes, 2014). Although, most *fru*-positive cells

express all three *fru* isoforms (Billeter et al., 2006), there is considerable variation in function and regulatory targets that can affect the elaboration of neural circuitry and behaviour. The distinctive DNA binding domains of these isoforms also confer the different regulatory roles they serve, and each *fru* isoform can potentially play a unique and integral role in behaviours such as species-specific female preference.

The role of the *fru* isoforms is not extensively studied in females, but the presence of *fru* isoforms originating from the sex non-specific promoters P2-P5 in the CNS of males and females and their putative role in the formation of axonal tracts and neural patterning (Song et al., 2002) makes it plausible that these isoforms might also control female reproductive behaviour.

In hybrids bearing the *fru* isoform-specific mutants, the *D. simulans fru* isoforms are unmasked and this is sufficient to induce the *simulans*-like rejection of *melanogaster* males. This indicates a lack of redundancy in their role in species-specific female preference, similar to the role these isoforms play in male courtship behaviour (Neville et al., 2014). Furthermore, rescued expression of only the Fru^{C} isoform is able to restore *D. melanogaster*-like receptivity, corresponding with the highly specific and essential role of Fru^{C} isoforms in various aspects of male courtship. The loss of receptivity in $fru^{\Delta A}$, $fru^{\Delta B}$, and $fru^{\Delta C}$ females indicate a specific role for each isoform that cannot be restored by the other isoforms, but the rescue of female receptivity upon expression of Fru^{C} isoforms contradicts the former observation. This is a conundrum that warrants further examination and likely hints at a much more complex system at work. Future experiments can be performed with split-*GAL4* drivers that will limit expression of these *UAS-fru* transgenes only in small subsets of cells can be used to identify potential neurons that govern species-specific preference.

3.5 Conclusion

The *fru* isoforms coding Zinc-finger DNA binding domains are highly conserved across multiple insect species (Bertossa et al., 2009; Clynen et al., 2011; Davis et al., 2000), this suggests a common function of *fruitless* with a possible ancient origin. There is also strong evidence of positive selection acting on the fru^{A} exons across multiple *Drosophila*

species (Neville et al., 2014), whereas the *fru*^{B} and *fru*^{C} exons are highly conserved and more likely to be necessary for essential functions. Therefore, understanding the function of these *fru* isoforms and how they affect female preference might be the key to identifying the genetic and neural mechanisms by which *fruitless* is influencing species-specific female preference.

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Chapter 4

4 Generating tools for studying behavioural isolation between *Drosophila melanogaster* and *Drosophila simulans*

4.1 Introduction

Reproductive isolating mechanisms emerge as barriers that prevent successful mating between individuals from different species (Mayr, 1942). Behavioural isolation is one of the earliest acting and strongest reproductive barriers to hybridization and gene flow between species (Coyne and Orr, 1997, 2004) and is considered to be an important factor in the speciation process. Behavioural isolation often manifests as incompatibilities in mating strategies resulting in a lack of mutual sexual attraction between individuals from different species (Coyne and Orr, 2004). Typically, males produce signals that elicit receptivity from conspecific females but not from heterospecific females. When male signals and female preference become discordant between prospective mates from different populations due to drift, it can result in assortative mating and, eventually, reproductive isolation (Ting et al., 2001; Coyne and Orr, 2004).

In *Drosophila*, and many other insect species, courtship rituals involve the exchange of visual, auditory, tactile and olfactory signals that can affect mating success (Spieth, 1974; Partridge et al., 1987). Many of these signals have components that affect species-specific recognition and thus potentially act as mating barriers between species. For example, the species-specific inter-pulse intervals in *Drosophila* "courtship song" has been shown to influence sexual isolation between species in the *Drosophila melanogaster* species group (Ewing and Bennet-Clark, 1968; Ritchie et al., 1999; Tomaru et al., 2000). Ultimately, divergence in female preference for this and other signals can contribute to species isolation. A small number of genomic regions that affect variation in species-specific female preference have been identified (Campesan et al., 2001; Fitzpatrick et al., 2005; Kronforst et al., 2006; Moehring et al., 2006; Laturney and Moehring, 2012a), these regions may contain genes that affect auditory or olfactory systems that influence mate discrimination, or genes that are involved in the processing of sensory information.

Variation in these genes between populations may cause a change in female preference and contribute to behavioural isolation between species (Noor et al., 2001; Nanda and Singh, 2011). Therefore, identification of genomic regions influencing female preference is an important and necessary step to uncover the genetic basis of behavioural isolation.

Extensive research on behavioural isolation has been conducted with species from the *Drosophila* genus, where multiple, closely related species exhibit mating isolation (Coyne, 1989; Nanda and Singh, 2012). The sympatric species pair *Drosophila melanogaster* and *Drosophila simulans* is of interest owing to the asymmetry of the isolation; female *D. simulans* rarely mate with *D. melanogaster* males (Sturtevant, 1920; Barker, 1967; Carracedo et al., 2000;) but *D. melanogaster* females mate with *D. simulans* males if placed in a no-choice assay, producing sterile hybrid females that are receptive to *D. melanogaster* males (Sturtevant, 1920; Manning, 1959). This pattern, where hybrids females behave like *D. melanogaster* and not *D. simulans* indicates that any potential loci that contribute to *D. melanogaster*-like receptivity for interspecific males are dominant or semi-dominant over the corresponding *D. simulans* loci.

Since F1 hybrid females between *D. melanogaster* and *D. simulans* are sterile, recombination mapping techniques, which rely on producing fertile hybrid offspring, are not possible. However, this limitation towards gene identification can be circumvented using deficiency mapping within hybrids (Pasyukova et al., 2000; Laturney and Moehring, 2012b). Owing to the vast number of deficiencies and the extensive genetic toolkit available for *D. melanogaster*, this species pair can be a powerful model for identifying genes that contribute to species-specific female preference and behavioural isolation.

A previous study by Laturney & Moehring (2012b) used deficiency mapping to identify several regions in the right arm of the 3rd chromosome that contain potential candidate genes (Laturney and Moehring, 2012a). A candidate region identified using this method was further mapped using smaller deletions within these regions to identify one specific gene of interest: *fruitless* (Moehring et al. Unpublished). Subsequent assays using the deficiency mapping approach with known *fruitless* disruptions (Lee et al., 2000; Anand et

al., 2001; Stockinger et al., 2005; Kimura et al., 2008, 2005) showed that disruption of the *D. melanogaster* allele of *fruitless* in F1 hybrids, leaving only the functional *D. simulans* allele, renders females less receptive towards *D. melanogaster* males. This gene-mediated loss-of-receptivity suggests that the *D. simulans fruitless* allele is affecting species-specific female rejection (Chapter 2).

In Chapter 2, I tested a number of *fru* disruptions using a genetic mapping approach similar to deficiency mapping to confirm that *fru* is indeed affecting species-specific preference. I found that, disrupting the *D. melanogaster fru* allele in hybrids results in a loss of receptivity to *D. melanogaster* males. Further, one of the *fru* disruptions that had a strong effect on species-specific female preference, $fru^{MI05459}$, showed reduced expression of transcripts starting with the *fru* P2 exon and an elimination of *fru* P5 expression. Excision of the disrupting *Minos* element rescued female receptivity to *D. melanogaster* males, and also rescued *fru* P2 and P5 expression. Using a precise deletion of *fru* P2, I was able to determine that *fru* P2 transcripts have a significant reduction in species-specific female rejection. However, it is possible that a P2 deletion might have a subtle effect on P5 expression, so the causal effect of P2 on female receptivity should be confirmed using an alternative approach, such as by knocking down P2 transcript expression using RNA interference (RNAi). The first reported discovery of RNAi molecules interfering with protein expression was observed in *Caenorhabditis elegans* (Fire et al., 1998). There are primarily three types of RNAi, (miRNA, siRNA and shRNA) that are all processed into 21-22 nucleotide double stranded RNA molecules (Elbashir et al., 2001). Micro-RNA (miRNA) molecules are processed into pre-miRNAs by the proteins Drosha and Pasha (Tomari and Zamore, 2005) and the pre-miRNAs are transported to the cytoplasm and processed by the protein Dicer into their mature form (Lee et al., 2002; Lund and Dahlberg, 2006). The RNA-induced silencing complex (RISC) is guided by the antisense strand in the mature miRNA, to its complementary mRNA sequence, which is then cleaved (Torrecilla et al., 2014). Short interfering RNA (siRNA) molecules are double stranded RNA constructs injected into the cytoplasm and processed by Dicer before they are incorporated into RISC. Short hairpin RNAs act in a similar manner to siRNA, but need nuclear processing similar to miRNAs (Carthew and Sontheimer, 2009; Ha and Kim, 2014). These are also synthetic double stranded RNA

molecules introduced into the nucleus, transcribed and processed into pre-shRNAs (Rao et al., 2009). These pre-shRNAs are then transported into the cytoplasm by Exportin 5 and processed by Dicer into siRNA and incorporated into RISC to bind to the complementary target mRNA. Both siRNA and shRNA require perfect complementarity to the target mRNA to silence expression of the transcript, but they can result in off-target effects when nucleotides in the 5' seed region are complementary to other mRNA transcripts (Lim et al., 2005).

There are advantages of using RNA interference (RNAi) to silence a specific transcript, and for silencing of the *fru* P2 transcript in particular. RNAi allows the transient manipulation of expression at specific developmental stages or in specific tissues, which permits an assessment of which time point this transcript acts to affect female behaviour. Additionally, owing to sequence variation between *Drosophila melanogaster* and *D. simulans fru* P2 and P5 exons, RNA interference can be used to silence either the *D. melanogaster* or *D. simulans* allele's transcripts of *fru* in F1 hybrids. If silencing the *D. melanogaster fru* P2 or P5 expression in hybrids, so that only the corresponding *D. simulans fru* transcripts are expressed, results in a reduction of receptivity to *D. melanogaster* males, it will confirm the effect of these transcripts on species-specific female preference. Similarly, reciprocal knockdown of *D. simulans fru* transcripts alone in hybrids should not result in a reduction in receptivity towards *D. melanogaster* males. This would be a definitive test, to show that the effect of *fru* on female preference is a species-specific one, and not just the result of a loss of one set of *fru* transcripts.

RNAi has been a widely-used technique for gene silencing in *D. melanogaster* (Dietzl et al., 2007), and there are a variety of resources available through the *Drosophila* RNAi Screening Center (DRSC) and Transgenic RNAi Project (TRiP), which both house huge libraries of established RNAi lines and vectors for designing RNAi experiments for a particular gene of interest (Dietzl et al., 2007; Ni et al., 2011). Some RNAi vectors, such as pVALIUM20 (Ni et al., 2011), have several upstream activating sequences paired to the heat sensitive promoter *Hsp70*, which allows expression of short hairpin RNAs in a time- and tissue-specific pattern. Temporal control can be achieved by exposing flies to high temperatures (28°C or 30°C) at a desired developmental stage, while pairing the

UAS-RNAi line with different tissue-specific *GAL4* drivers allows spatial control of the RNAi expression via the *GAL4/UAS* system.

The *GAL4/U*AS system is a versatile tool for targeted gene expression in *Drosophila*. The *GAL4* protein is a yeast transcriptional factor that binds to an upstream activating sequence (*UAS*) to activate expression of any gene of interest downstream of the *UAS* (reviewed in Duffy, 2002). The *GAL4* expression is controlled by nearby promoters or enhancers, as a result the *GAL4* protein is produced in a tissue specific pattern that is consistent with the expression of the promoter or enhancer (Brand and Perrimon, 1993). This allows for precise spatial and temporal expression of any gene of interest (i.e. reporter genes) linked to *UAS* motifs (Brand and Perrimon, 1993). In *D. melanogaster*, there is a vast collection of transgenic lines with *GAL4* drivers inserted in the genome whose expression pattern has already been characterized. Likewise, there are numerous lines containing *UAS* sequence paired with reporter transgenes. Crossing a particular *GAL4* driver with a *UAS* linked gene of interest produces offspring with both *GAL4* and *UAS* elements, driving defined expression of the transgene, which can be used to test the effect of the gene of interest in specific tissues.

In this chapter, I will outline the development and testing of transgenic *UAS-shRNAi* lines targeting either the *D. melanogaster* or *D. simulans* allele of the *fruitless* P2 or P5 transcript. By inducing expression of these *UAS-shRNAi* constructs with three different *GAL4* drivers, I will knockdown either the *D. melanogaster* or *D. simulans fruitless* P2 or P5 transcripts in hybrids. I will use RT-PCR to validate the knockdown of these transcripts, and mating assays to test if silencing the expression of the *D. melanogaster* or *D. simulans* P2 or P5 transcripts in hybrids has an effect on species-specific female preference.

4.2 Methods

***Drosophila* Strains and Crosses:** Flies were maintained on standard food medium (Bloomington *Drosophila* Stock Center) under a 14:10 light:dark cycle at 24ºC and approximately 80% relative humidity. All the *GAL4* and *UAS-shRNAi* constructs were maintained over 2nd or 3rd chromosome balancers *CyO, TM3* or *TM6C*, which contain a

dominant visible phenotypic marker and serial inversions preventing recovery of recombinant offspring between the homologous chromosomes. Unless otherwise noted, all stocks were obtained from the Bloomington *Drosophila* Stock Center (Bloomington, Indiana). A balancer stock (w^{1118}/*Dp(1;Y)*y^{+}*; CyO/nub*^{1} *b*^{1} *sna*^{Sco} *lt*^{1} *stw*^{3}*; MKRS/TM6B,* *Tb*^{1}) is used to introduce 2nd and 3rd chromosome balancers. Several *GAL4* drivers were used to drive expression of the UAS-shRNA constructs, including a ubiquitous *GAL4* driver (y^{1} w^{1}*; P{w[+mC]=Act5C-GAL4}17bFO1/TM6B,* *Tb*^{1}), and two *fruitless GAL4* drivers (w^{1118} *;P{w[+mW.hs]=GawB}fru*^{NP0021}) and (*w*; TI{GAL4}fru*$^{GAL4.P1.D}$*/TM3,* *Sb*^{1}, provided by Dr. Barbara Taylor). A *D. melanogaster* stock with GFP-tagged sperm (*w; P{w8, ProtA-EGFP,* w^{+}*}19B(3)*) was provided by Dr. John Belote. The wild-type *D. melanogaster* BJS is an isofemale line collected by Dr. Brent Sinclair and wild-type *D. simulans* Florida City (FC) was provided by Dr. Jerry Coyne (collected from Florida City, Florida; Coyne, 1989). The transgenic shRNAi lines were created by injecting the short hairpin RNAi constructs into embryos from a stock expressing phiC31 (ΦC31) integrase in the germ line and which also contains an *attP* site in the left arm of the third chromosome (y^{1} v^{1} *P{nos-phiC31\int.NLS}X; P{CaryP}attP40*).

Preparing short hairpin RNA constructs: Short hairpin RNAs were designed to target either the *D. melanogaster* or *D. simulans fru* P2 or *fru* P5 exons. Both exons have single nucleotide polymorphisms between the two species, which was confirmed by sequencing (Chapter 2). By targeting regions of nucleotide variation within the exons, it is possible to design shRNAs that will only silence transcripts from one particular species. Two separate *D. melanogaster* and *D. simulans* shRNA constructs were created for both P2 and P5 (eight constructs in total). The shRNA constructs were cloned into pVALIUM20, which contains upstream activating sequences (*UAS*) paired with an *Hsp70* heat-sensitive promoter (Ni et al., 2011). This allows the temporal and spatial control of shRNA expression by exposing flies to high temperatures (28°C) at desired developmental stages and by activating expression using different tissue-specific *GAL4* drivers (Ni et al., 2011). The vector also contains an *attB* site that allows for site specific integration using ΦC31 integrase (Groth et al., 2004). The shRNA constructs were cloned into pVALIUM20 using the protocol from (Chang et al., 2014), and the recombinant pVALIUM20 vectors were used to transform 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 prior to plating on LB+ampicillin plates (100μL/mL) and growing them overnight at 37°C. Four colonies were selected for each shRNA construct and the plasmid was extracted using PureLink™ Quick Plasmid Miniprep Kit (Cat# K210010). The extracted plasmid DNA from the colonies were PCR amplified using the following primers: pVAL_SelF: ACCAGCAACCAAGTAAATCAAC, and pVAL_SelR: TAATCGTGTGTGATGCCTACC, to verify the transformations. Successful transformants produced a 350bp PCR product, which was verified by sequencing, whereas unsuccessful colonies gave a 900bp product.

Microinjection protocol: One week prior to injections, approximately 500 flies from the phiC31 (ΦC31) integrase stock (*y*^{1} *v*^{1} *P{nos-phiC31\int.NLS}X; P{CaryP}attP40*) were transferred into an embryo cage with egg laying plates and kept at 25°C and 70% humidity. Embryos were harvested on apple juice agar plates topped with fresh yeast paste that were changed twice each day until the day of injections. On the day of injection, the egg laying plates were changed every 30 minutes for at least four hours prior to the start of injections, to flush older embryos withheld by females. Freshly laid embryos were washed off of the agar plates into a clean mesh basket, transferred onto a coverslip, aligned using a moist paintbrush, and coated with extra virgin olive oil

Microinjection needles were prepared in advance using 1.0mm borosilicate microcapillary tubes that were pulled using a Micropipette P-97 needle puller (Sutter Instrument Company). The microinjection needles were filled with the injection mix using loading needles pulled from 1.0 mm borosilicate microcapillary tubes over a Bunsen burner. The injection mix was prepared by mixing a 10 ul aliquot of the shRNA plasmid and 2 ul of blue food colouring. The aligned embryos were injected at the posterior end using a Sutter Instruments microinjector and the olive oil was washed off immediately after injections. Injected embryos are placed in a petri dish with standard food medium (Bloomington *Drosophila* Stock Center) and stored at 24ºC and 80% relative humidity under a 14:10 light:dark cycle until pupation. The pupa were then transferred to food vials until the adults eclosed.

Screening and crosses: Injected G_0 adults were backcrossed to the parental stock (y^1 v^1 *P{nos-phiC31\int.NLS}X; P{CaryP}attP40*) and the offspring from these crosses were screened for eye colour. Transgenic flies were identified by their wild-type eye colour due to the presence of *vermillion+* in the insertion, while flies with mutant *vermillion* eyes were discarded. Virgin transgenic females were crossed with the males from the balancer stock (w^{1118}*/Dp(1;Y)*y^+*; CyO/nub*^{1} b^1 sna^{Sco} lt^1 stw^3*; MKRS/TM6B,* Tb^1) to introduce balancers into the genetic background and create stable balanced *UAS-shRNA* stocks for both *D. melanogaster* and *D. simulans* P2 transcripts (*UAS-MelP2shRNA* and *UAS-SimP2shRNA*). I was unable to inject the shRNA constructs targeting the *D. melanogaster* and *D. simulans* P5 exons due to time constraints.

Virgin females from different *GAL4* lines (*Act5C-GAL4,* fru^{GAL4}, and fru^{NP0021}) were crossed with the balancer stock to introduce balancers into the *GAL4* stocks. These balanced *GAL4* stocks were crossed with the balanced UAS-shRNA stocks to produce flies that contain both *GAL4* and *UAS-shRNA* constructs.

Mating Assays: Five or six virgin females from balanced stocks with both *Act5C-GAL4* and *UAS-MelP2shRNA* or *UAS-SimP2shRNA* were aged five to seven days and then crossed with 20-25 non-virgin *D. simulans* FC males to produce F1 hybrid females with both *Act5C-GAL4* and *UAS-MelP2shRNA* or *UAS-SimP2shRNA* (*GAL4+UAS*), raised at 28ºC. Separately, 5-6 virgin females from balanced stocks with only the *Act5C-GAL4* or only the *UAS-MelP2shRNA* or *UAS-SimP2shRNA* were aged 5-7 days and crossed with approximately 20 non-virgin *D. simulans* FC males; these crosses were raised at 28ºC as above. These hybrids will only have the *Act5C-GAL4* (*GAL4*) or only the *D. melanogaster* or *D. simulans UAS-P2shRNA* constructs (*UAS BAL*), and will serve as controls

Virgin hybrid females of the three genotypes produced above were paired with a 5-7 day old virgin *D. melanogaster* GFP-sperm male in a no-choice mating assay (as in Austin et al., 2014). Assays were carried out at 28ºC, ~70% relative humidity, and 1-2 hours of "lights on" in 30mL plastic vials containing approximately 5 mL of standard food medium. Flies were left within the vials for 24 hours, at which time the female was

decapitated and her reproductive tract dissected. The reproductive tracts were imaged using a compound fluorescent microscope and scored for the presence or absence of fluorescently-labelled sperm as a proxy for copulation occurrence. The mating proportion of flies from the genotypes were compared using a *Z*-test ($\alpha < 0.05$).

RT-PCR: To test the efficacy of the *D. melanogaster fru* RNAi, RNA was extracted from 20 adult *D. melanogaster* females (5-7 day old) with both *GAL4* and *UAS-shRNA* constructs, that were raised at 28ºC until 5-7 days following eclosion. To test the species-specificity of *D. melanogaster fru* RNAi, and to test *D. simulans fru* RNAi, interspecies hybrids were assayed. For the interspecies crosses raised at 28ºC, F1 hybrids bearing both *Act5C-GAL4* and *UAS-P2shRNA* constructs did not survive past pupation and so adults were not available to test the effects of silencing P2 expression. For these crosses, RNA was extracted from 10-15 pupae instead.

Total RNA was extracted using the TRIzol plus Purelink RNA purification kit (Thermofisher Cat# A33254), RNA was quantified using a Nanophotometer P300 (Implen, Inc.), and 2 ug of total RNA was used for cDNA synthesis using Maxima First Strand cDNA Synthesis Kit with DsDNAase (Thermofisher, Cat# K1671) using oligo dTs. RT-PCR was performed to check for presence of *fru* P2, P5, P3 and P4 transcripts using a forward primer within the exon (P2-F: AATCGTCGCGGTCATAAAAT; P5-F: ACATAGACAGTGCCTCGT; P3-F: TCATCAGCAAATGCCTCGT; P4-F: CCAAAAACTAAGCCCGTCAA) and a reverse primer within the common region of *fru* (COM-R: AGTCGGAGCGGTAGTTCAGA) (see Table S4-1). *RpL32* was used as a control to compare gene expression levels (Rpl32-F: GGCATCAGATACTGTCCCTTG; Rpl32-R: CCAGTCGGATCGATATGCTAA). I used ImageJ software to compare the intensities of the bands and extrapolate the level of gene expression. Since hybrids have both *D. melanogaster* and *D. simulans* copies the *fru* P2 transcripts, the RT-PCR reactions will amplify both transcripts. To detect the presence of only the *D. simulans* P2 transcripts, the RT-PCR products were digested with BseY1 restriction enzyme, which digests the *fru* P2 transcripts from *D. simulans* but not *D. melanogaster.* Therefore, if both species' P2 transcripts are present, three bands will be observed on the agarose gel:

two bands (50 bp and 414 bp) from the *D. simulans* allele and one band (464 bp) from the *D. melanogaster* allele.

4.3 Results

All of the shRNA constructs I designed were successfully transformed into vectors. PCR verification of the colonies transformed with *D. melanogaster* and *D. simulans* P2 and P5 shRNA constructs showed 350bp bands, indicating successful transformation with pVALIUM20 (Figure 4-1). The PCR products were then extracted, gel purified and sequenced. The sequences confirmed successful cloning of the shRNA constructs into the pVALIUM20 plasmid. Due to time constraints, I was only able to inject embryos and successfully create *UAS-shRNA* lines targeting the *fru* P2 exons from *D. melanogaster* and *D. simulans*, but was not able to inject the *fru* P5 constructs. PCR verification of the genomic DNA from the *fru* P2 transgenic shRNA lines produced the expected 350 bp band, indicating successful integration of the pVALIUM20 and shRNA constructs into the genome (Figure 4-2).

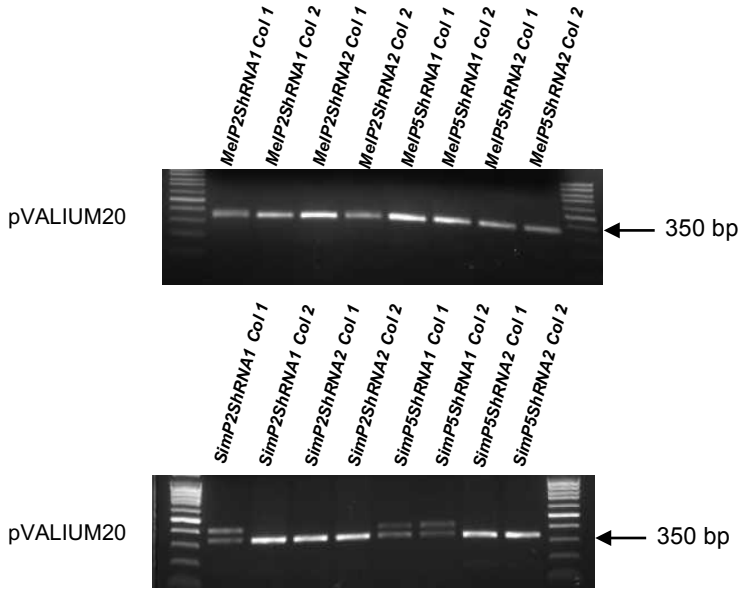

Figure 4-1 PCR detection of pVALIUM20 in transformed *E. coli* colonies. Successful transformation of *D. melanogaster* and *D. simulans* P2 and P5 shRNA constructs into *E. coli* 10 beta- competent cells, grown in LB+ampicillin. A 350 bp PCR product indicates successful transformation with *UAS*-P2shRNA constructs into *E. coli.*

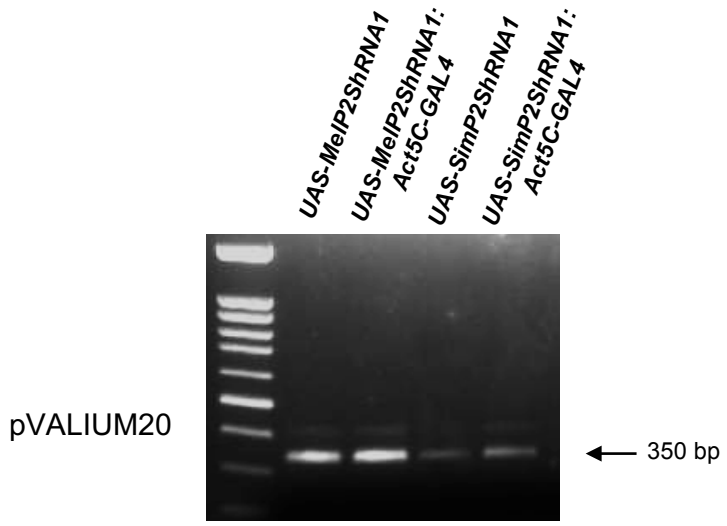

Figure 4-2 PCR detection of pVALIUM20 in *D. melanogaster* (*UAS-MelP2shRNA*) and *D. simulans* (*UAS-SimP2shRNA*) shRNA stocks. A 350 bp PCR product indicates the presence of the pVALIUM 20 shRNA construct in the *D. melanogaster* transgenic stocks for *UAS-MelP2shRNA* and *UAS-SimP2shRNA* constructs. The 350 bp pVALIUM20 PCR product is also present in the *Act5C-GAL4: UAS-MelP2shRNA* and the *Act5C-GAL4; UAS-simP2shRNA* transgenic stocks.

The results of the RT-PCR indicate reduced expression of *D. melanogaster fru* P2 when *UAS-MelP2shRNA* expression is driven by the *Act5C-GAL4, fru*^{NP0021} and *fru*^{GAL4}. Analysis of the band intensities showed an approximate 80% reduction in P2 expression using the *Act5C-GAL4* and *fru*^{NP0021}*GAL4* drivers and a 50% reduction using the *fru*^{GAL4} driver. P2 expression is only reduced by 27% when P2 expression is silenced during the adult stages (Figure 4-3). P2 expression in *Act5C-GAL4:UAS-SimP2shRNA*, line was comparable to that of the wild-type *D. melanogaster fru* P2 expression and the *Rpl32* controls. *Act5C-GAL4:UAS-MelP2ShRNA* hybrids raised at 28ºC were lethal. Approximately 30 different vials of crosses were set up at 28ºC to produce these interspecies hybrids, but none of the *Act5C-GAL4:UAS-MelP2ShRNA* or *Act5C-GAL4:UAS-SimP2ShRNA* hybrids survived past the pupal stages when raised at 28ºC. These hybrid pupae showed an approximate 70% reduction in P2 expression compared to *D. melanogaster, D. simulans* and wild-type hybrids. but there was no notable reduction in the expression of P3, P4 and P5 transcripts compared to *D. melanogaster*, *D. simulans*

and wild type hybrids (Figure 4-4). The effect of the *D. simulans* P2 RNAi is unclear, since *Act5C-GAL4:UAS-SimP2shRNA* hybrids showed reduced P2 expression (~ 60%) compared to wild-type hybrids and wild-type *D. simulans* females (Figure 4-5). However, although the expected 414 bp band was produced in wild-type *D. simulans*, I did not observe the expected 414 bp product in the *Act5C-GAL4:UAS-SimP2shRNA* hybrids after digesting the P2 RT-PCR product with BseY1 restriction enzyme (Figure S4-1).

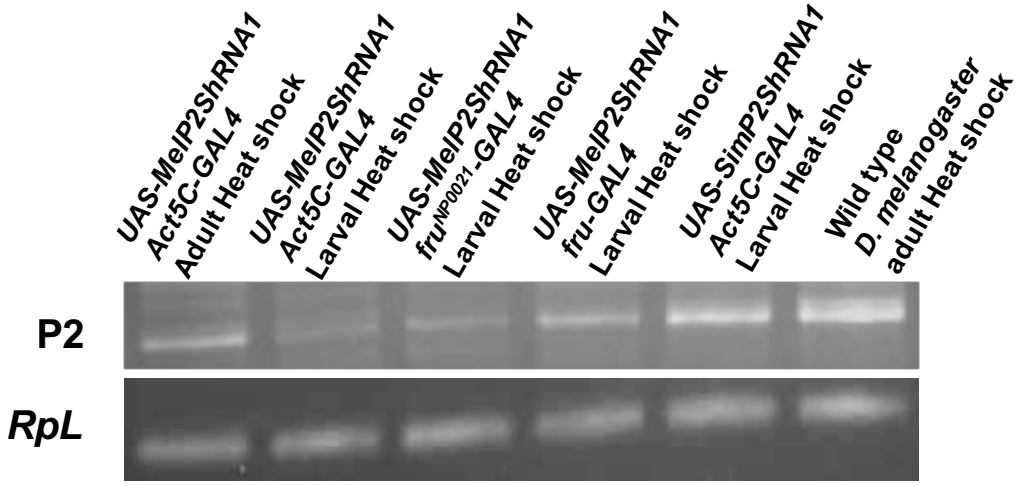

Figure 4-3 RNAi knockdown of *fru* P2 expression in *D. melanogaster UAS-Mel* P2 shRNA transgenic flies using different *GAL4* drivers. *UAS-MelP2shRNA or UAS-SimP2shRNA* constructs were activated using either *Act5C-GAL4, fru*^{NP0021} and *fru*^{GAL4} drivers by heat shocking from larval stages or after eclosion as adults. RNA was extracted immediately after heat shock treatment. P2 expression was compared to wild-type *D. melanogaster* females heat shocked after they eclosed. *RpL32* was used as a control for expression.

Due to lethality, I was unable to assess the effects of silencing *fru* P2 expression during all developmental stages. However, lethality was not observed when *Act5C-GAL4:UAS-P2shRNA* hybrids were raised at 24ºC, or in F1 hybrids bearing only the *Act5C-GAL4* or the *D. melanogaster* or *D. simulans UAS-P2ShRNA* constructs, when raised at 28ºC or 24ºC.

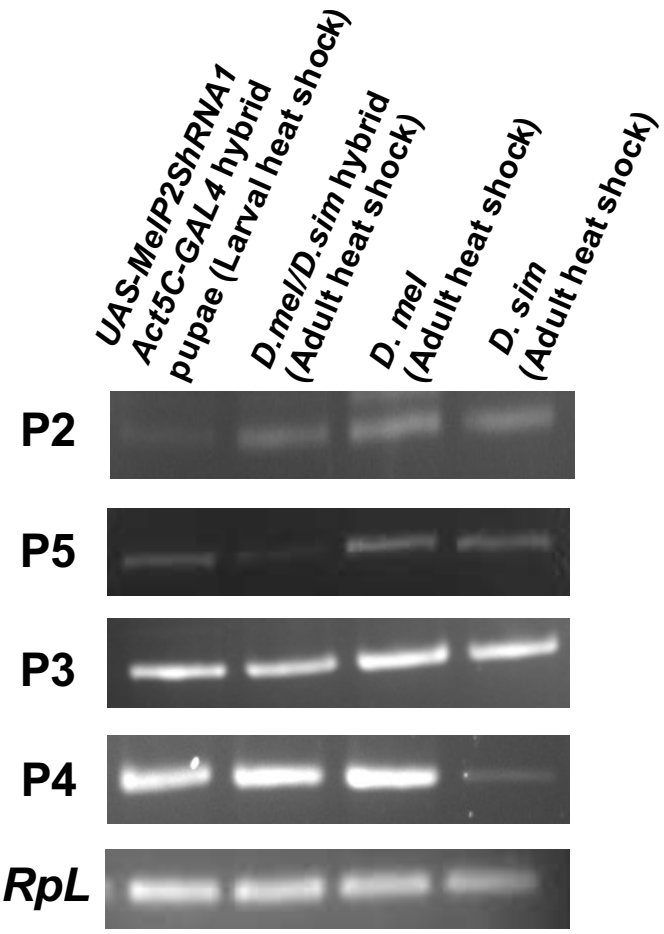

Figure 4-4 RT-PCR to detect knockdown of *fru* transcripts in *Act5C-GAL4:UAS-MelP2shRNA* hybrid pupae heat shocked from early larval stages. P2 expression in hybrid *Act5C-GAL4:UAS-MelP2shRNA* pupae is lower compared to *D. melanogaster/D. simulans* hybrids, wild-type *D. melanogaster* females or wild-type *D. simulans* females that are heat shocked post eclosion. Expression of P5, P3 and P4 in these *Act5C-GAL4:UAS-MelP2shRNA* hybrids appear to be comparable to wild-type hybrids and wild-type *D. melanogaster* and *D. simulans* females. *RpL32* was used as a control for expression.

Therefore, I used these hybrids in mating assays to assess the effect of silencing P2 expression on female receptivity. *Act5C-GAL4:UAS-MelP2shRNA* (*GAL4+UAS*) did not show a significant reduction in mating proportion compared to hybrids with only the *Act5C-GAL4* (*GAL4*) ($z = 1.581$; $p = 0.114$; $n = 20$) or the *UAS-MelP2shRNA* flies (*UAS BAL*) ($z = 0.886$; $p = 0.373$; $n = 20$) (Table 4-1). There was also no difference in mating

proportion between *Act5C-GAL4*:*UAS-SimP2shRNA* hybrid females, and hybrids with only the *Act5C-GAL4* (*GAL4*) (z = 0.358; p = 0.719; n = 19) or the *UAS-SimP2shRNA* hybrids (*UAS BAL*)(z = 0.698; p = 0.484; n = 19) (Table 4-1). This indicates that silencing *D. melanogaster* P2 expression in adults is not sufficient to induce *D. simulans*-like rejection in female hybrids.

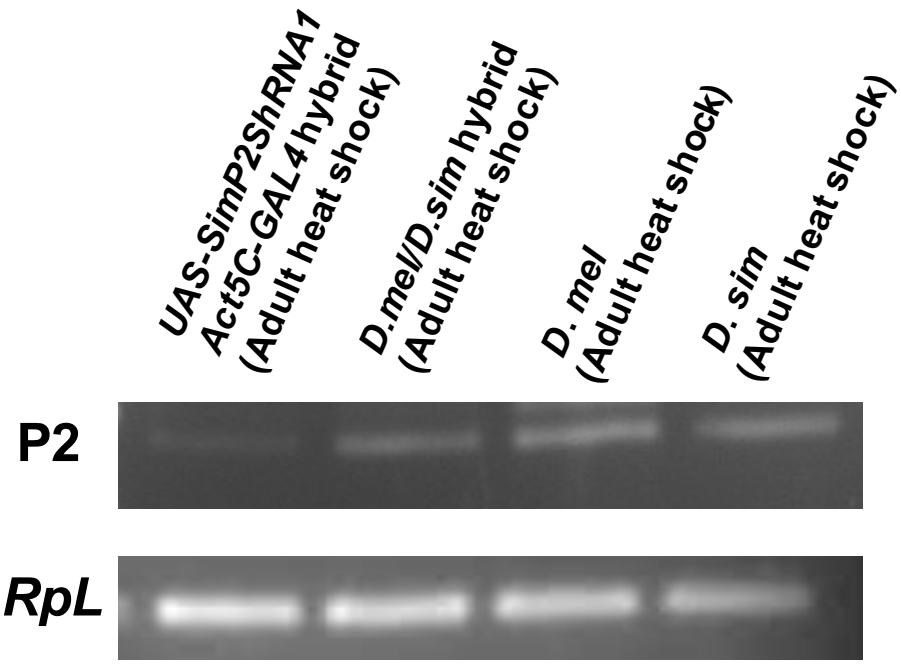

Figure 4-5 RT-PCR to detect knockdown of P2 transcripts in *Act5C-GAL4:UAS-SimP2shRNA* hybrid females heat shocked post eclosion. P2 expression in these hybrid females appear to be lower than *D. melanogaster*/*D.simulans* hybrids, and wild-type *D. melanogaster* and *D. simulans* females. *RpL32* is used as a control for gene expression.

Table 4-1 Behavioural assays with *D. melanogaster* and *D. simulans fru* P2 RNAi knockdown hybrid females (*Act5C-GAL4:UAS-MelP2shRNA* and *Act5C-GAL4:UAS-SimP2shRNA*) with *D. melanogaster* males at 28^{0}C. The proportion of mated hybrid females with both *GAL4* and *UAS* constructs (*GAL4+UAS*) is compared to the proportion of mated females bearing only the *GAL4* or the *UAS* constructs using a *Z*-test $\alpha < 0.05$.

				sim/mel $^{GAL4+UAS}$			*sim/mel* ^{GAL4}			*sim/mel* $^{UAS\ BAL}$			GAL4+UAS vs GAL4		GAL4+UAS vs UAS BAL	
Name	*D. melanogaster* genotype used to make hybrid females	GAL4 driver	N	Court	1h Cop	24h Cop	Court	1h Cop	24h Cop	Court	1h Cop	24h Cop	Z	*P*-value	Z	*P*-value
MelP2shRNA1	*w; UAS-melP2shRNA1 ; Act5C-GAL4/TM3, ftz-lacZ*	*Act5C-GAL4*	20	20	0	2	19	2	6	20	1	4	1.5811	0.114	0.8856	0.373
SimP2ShRNA1	*w; UAS-simP2shRNA1 ; Act5C-GAL4/TM3, ftz-lacZ*	*Act5C-GAL4*	19	18	0	6	17	0	6	16	3	7	0.3577	0.719	0.698	0.484

4.4 Discussion

The reduced expression of the P2 transcripts suggests that the *MelP2shRNA* constructs are silencing *fru* P2 expression, but not eliminating P2 expression completely. Therefore, a qualitative assessment such as RT-PCR might not be sensitive enough to evaluate the efficacy of the RNAi knockdown and it should be tested using a quantitative approach such as qPCR. Additionally, a similar knockdown is not observed in *D. melanogaster* females with the SimP*2shRNA* constructs, which suggests that the *D. simulans* P2 shRNA is not targeting the *fru* P2 transcript in *D. melanogaster*.

The lethality observed in *Act5C-GAL4:UAS-MelP2ShRNA* hybrids raised at 28ºC suggests potential off-target effects, but the presence of *fru* P3, P4 and P5 transcripts in these hybrid pupae raised at 28ºC discounts the possibility of unintended silencing of these other *fru* transcripts, two of which (P3 and P4) are known to cause lethality when disrupted (Anand et al., 2001; Song et al., 2002). As *fru* P2 is not known to affect adult viability (Anand et al., 2001), there might still be potential RNAi off-target effects that silence the expression of genes essential for adult viability. However, since P2 RNAi expression is not affecting viability in pure species females, this off-target effect would have to act in the *D. simulans* genome. Alternatively, it could be due to more complex interactions between this silencing and epistatic interactions involving the two species' genomes, negatively affecting hybrid fitness. There is evidence supporting the presence of these types of negative interactions, with multiple genes inducing hybrid inviability due to epistasis between the genomes of these two species (Barbash et al., 2000). This lethality might be circumvented by using tissue-specific *GAL4* drivers to limit the expression of the shRNA constructs only to neurons or subsets of neurons, or by activating expression of the shRNA only during specific developmental stages. The caveat of this approach is that the developmental stage where the RNAi is causing lethality could also be the stage where *fru* transcripts are acting to cause an effect on female behaviour. Therefore, limiting the RNAi expression to only specific stages during development might not produce an effect in female receptivity. Another possible explanation is that the loss of P2 expression combined with the stress of heat shock might have a more extreme effect in hybrids than in pure species females, which might

contribute to hybrid lethality. Follow up experiments using hybrids with a *fru* P2 deletion (Chapter 2) raised at 28ºC can be an effective approach to test this theory.

These results suggest that the RNAi knockdown of *D. melanogaster* P2 transcripts in adults might not be sufficient to impact species-specific female receptivity. This suggests that the effect of P2 on female receptivity is either developmental (e.g. affecting neural patterning) or that the incomplete nature of RNAi, which may allow a small amount of transcripts to still be present, is sufficient for female behaviour. Since I did not observe any significant difference in female receptivity for *Act5C-GAL4:UAS-SimP2shRNA* hybrids and the control hybrid females, it is possible that the knockdown of *D. simulans* P2 transcripts do not affect the female's receptivity to *D. melanogaster* males. However, because I was unable to verify that the *SimP2shRNA* constructs are indeed silencing *D. simulans* P2 expression, it is not possible to confirm the effect of silencing the *D. simulans fru* P2 transcripts on female receptivity. Based on these results I am unable to confirm that female receptivity to interspecies males is an allele specific effect and not a result of hemizygosity.

The results of the behavioural assays and the expression data, suggest that the P2 shRNA constructs are silencing the expression of *fru* P2 in a subtle manner. Due to the lethality observed in hybrids it was not possible to determine the effect of constitutive expression of the P2 shRNA constructs during all developmental stages. It is therefore possible that P2 expression is necessary at specific developmental stages to have an effect on species-specific female receptivity. Silencing P2 expression at specific developmental stages and testing the effect of the knockdown using behavioural assays can be a more effective approach to evaluate the role of P2 in female receptivity and to identify the developmental stages during which P2 expression influences female receptivity. Since I did not observe a decrease in female receptivity in hybrids with *MelP2ShRNA* but only with *SimP2ShRNA,* the role of *fru* P2 expression on species-specific female receptivity cannot be confirmed with certainty. An alternative approach using CRISPR to create deletions of *D. simulans* P2 transcripts is likely to produce more definitive results about the species-specific nature of the effect of *fru* P2. However, RNAi knockdown offers a more systematic approach for testing the effects P2 transcripts, through the use of tissue-

specific *GAL4* drivers that can identify regions of the brain or specific neuronal classes that might be involved in species-specific female receptivity.

Although pVALIUM20 is known to induce strong silencing of target mRNA with considerable reduction in the expression of the target mRNA, it is known to result in unintended leaky expression resulting in an abundance of short hairpin RNA that could also produce off-target effects through fortuitous recognition of mRNAs via the 'seed' region and this can lead to unexpected lethality (Ni et al., 2011). This leakiness can also make it difficult to maintain stable RNAi lines that target genes that can affect viability (Ni et al., 2011), something that should not have been a factor for *fru* P2. However, this vector is well-characterized as an effective vector for RNA interference and allele specific gene silencing. This tool can be a viable alternative to gene editing techniques such as CRISPR, especially in the case of species that do not have an extensive transgenic toolkit available.

4.5 Conclusion

I was able to successfully generate transgenic RNAi lines targeting *D. melanogaster* and *D. simulans fru* P2 transcripts and confirm the knockdown using RT-PCR, but based on the results of the mating assays, the RNAi knockdown does not appear to have a significant effect on female receptivity. However, it is necessary to test the effect of the RNAi knockdown more extensively, by silencing P2 expression at different developmental stages, and also by using different *GAL4* drivers that express the P2 shRNA constructs in different tissues. I have yet to create transgenic shRNA lines for the *fru* P5 transcript, but I did create *fru* P5 shRNA vectors that are ready to be injected and screened for transformants prior to crossing in balancers and *GAL4* drivers. These RNAi lines can be very useful tools for determining the role of different *D. melanogaster* and *D. simulans fru* transcripts on female receptivity and can also be used to identify specific developmental stages or brain regions that have an important role in the development in species-specific female receptivity between *D. melanogaster* and *D. simulans.*

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Chapter 5

5 General Discussion

5.1 Introduction

The genetic basis of behavioural isolation among *Drosophila* is important to our understanding of how selection shapes species boundaries. Generally, multiple loci are involved in shaping the co-evolution of signals and signal preferences between the sexes. There is considerable evidence of functional genetic variation between *Drosophila* species, and while genes such as *period*, *doublesex*, *fruitless* and *chaste* are known to affect different aspects of mating behaviour within single species (Reviewed in Sokolowski, 2001), it remains unclear whether these same loci contribute to behavioural isolation between species. The idea that the same loci might influence both intra- and interspecific female preference is valid (Carracedo et al., 1989), but recent studies suggest that species-specific female preference is more likely determined by the action of multiple genes that are not involved in intra-specific preference (Gleason and Ritchie, 2004; Gleason et al., 2009; Laturney and Moehring, 2012a). The involvement of multiple genes was further supported by the findings of Laturney and Moehring (2012b) when they tested *dissatisfaction* and *spinster* for their effect on interspecific receptivity. They found that by muting each gene resulted in a significant reduction in female receptivity towards intra-specific males, but these same genes did not affect female receptivity towards interspecific males. Laturney and Moehring (2012b) also identified several genomic regions within the right arm of the third chromosome, with potential candidate genes for behavioural isolation between *D. melanogaster* and *D. simulans.* Subsequent fine-mapping within these regions identified a small genomic region containing a strong candidate gene: *fruitless.*

5.2 Thesis summary

Using multiple *fruitless* (*fru*) mutations, I was able to confirm that *fru* influences species-specific female receptivity between *D. melanogaster* and *D. simulans* (Chapter 2). Additional testing revealed that the effect of *fru* on female preference is not only species-specific, but also persistent across different strains of *D. simulans* (Chapter 2). The *fru* gene encodes transcription factors with closely related BTB (*Broad complex, Tramtrack* and *Bric-a-brac*) zinc-finger DNA binding domains (Ito et al., 1996; Ryner et al., 1996). The gene produces at least 15 different transcript variants, through different sites of transcription initiation and alternative splicing at the 3' end (Goodwin et al., 2000; Ito et al., 1996; Ryner et al., 1996; Song et al., 2002). Each *fru* transcript begins with one of five first exons (P1-P5), resulting in five different isoform groups of *fru* proteins. These proteins end with one of four distinct C-terminal zinc finger (ZnF) domains, three of which (Fru A-C) are predominantly expressed in the central nervous system (Baker et al., 2001).

To determine whether a specific group of transcripts is responsible for influencing species-specific female preference, I tested precise deletions of the P1 and P2 *fru* first exons and found that only the *D. simulans* P2 transcripts have a significant effect on species-specific female receptivity. Of note, the complete loss of P2 transcripts within *D. melanogaster* also caused a slight, but significant, reduction on female receptivity within species (Chapter 2). This is the first identified behavioural role of a non sex-specific *fru* transcript and the first identification of a gene that affects both intra- and interspecific female preference. Since *fruitless* is a highly pleiotropic gene and an important determinant in the formation of sexually dimorphic neural circuitry (Ito et al., 2012; Kimura et al., 2005; Lee et al., 2000), genetic variation can result in subtle changes in how the gene functions. This, in turn, can affect the development of reproductive behaviours. Genetic variants can be due to genetic drift or the result of adaptation (Hua and Bromham, 2017). Since the deletion of *fru* P2 results in a reduction in within-species female receptivity, *fru* P2 proteins likely have an essential function in this trait. It is possible that selection may act on this within-species role and lead to the eventual divergence in the sequence of this gene between two species. Upon analysis of the *D.*

melanogaster and *D. simulans fru* P2 sequence, I identified two adjacent SNPs that are predicted to cause a glutamate-to-glycine amino acid substitution at the second residue of *fruitless* P2 proteins. Based on the functional and structural differences between these two amino acids, this amino acid variation substitution can potentially have a drastic effect on the protein's function, (Betts and Russell, 2003; Purdue et al., 1992) and might be a possible explanation for how variation in the *fru* P2 exon can affect species-specific female preference between *D. melanogaster* and *D. simulans.*

At the 3' end of the *fru* gene, mutations in any of the 3' A, B or C exons also produced a significant effect on female receptivity. However, only expression the of *UAS-fru*^{C} (Song et al., 2002) isoforms using a *fru*-specific *GAL4* driver was able to restore female receptivity to wild-type levels (Chapter 3). Based on these findings, it appears that the *fru* P2 transcript with the *fru*^{C} 3' end is most likely influencing species-specific female preference. Since the drivers may not recapitulate the exact expression pattern of each isoform, we cannot rule out isoforms ending in A or B as potentially impacting female receptivity. Additionally, the causal effect of the C isoform cannot be confirmed without additional rescue experiments - for example, by driving expression of the *fru* P2 isoforms with the *fru*^{C} 3' end. This proposed experiment would require the creation of *UAS-fru* isoforms with specific 5' and 3' ends. If they can be generated, then I predict that the P2 and C exons would rescue female behaviour when ubiquitously expressed. These *UAS-fru* constructs can also be used in combination with different *GAL4* drivers for expression in specific tissues or subsets of neurons, which can be a used to identify and characterize different neurons or regions of the brain that might influence species-specific female preference in the context of the *fruitless* gene. The *GAL4/UAS* and *LexA/lexAop* binary systems, and FLP recombinase, are genetic tools that have been used to test subsets of *fru* neurons and identify neuro-anatomical dimorphisms, differences in interconnectivity and neural processing pathways (Billeter and Goodwin, 2004; Kimura et al., 2005; Manoli et al., 2005; Stockinger et al., 2005). Comparisons between the male and female *fru* expressing neural elements also identified distinct sexually dimorphic neuronal clusters, and arborization patterns that may contribute to the development of sex-specific behaviours (Cachero et al., 2010; Kimura et al., 2005; Mellert et al., 2010; Stockinger et al., 2005).

5.3 Neural basis of species-specific female preference

Although the neural circuitry involved in female post mating behaviour have been well characterized (Rezával et al., 2012, 2014; Yang et al., 2009), and few specific neurons that regulate female receptivity within species have been identified (Tompkins and Hall, 1983; Zhou et al., 2014), the higher-order neural circuits involved in species-specific female preference remains unknown. Previous experiments by a former student in our lab used *UAS-dTrpA*^{ts} (*Drosophila Transient receptor potential cation channel A1*) to hyperactivate or *UAS-shi*^{ts} (*shibire*) to silence specific regions of the brain using tissue specific *GAL4* drivers, to determine their role in intra- and inter-specific female preference (Mahabir and Moehring, Unpublished). Although no individual region affecting species-specific female receptivity was identified, this approach can be refined by using the intersectional split-GAL4/UAS system (Dionne et al., 2018), to limit expression of *UAS* transgenes to single neurons or specific combinations of *fruitless* positive neurons. These females can then be paired with males to determine these neurons' effect on species-specific female preference.

5.4 Sensory modalities that determine inter-specific female preference

Drosophila melanogaster and *D. simulans* have species-specific differences in mating cues that can play a role in behavioural isolation between these two species (Capy and Gibert, 2004). Removal or alteration of individual components of male courtship does not restore female receptivity to interspecific males (Ritchie et al., 1999; Tomaru et al., 2000). Presumably because other courtship components are still interspecific and the presence of a single negative courtship signal might be sufficient to induce female rejection. However, it is possible that the rejection of interspecific males in the context of *fruitless* may result from the processing of a single component of courtship, and removal of that component might rescue the receptivity to interspecific males. Therefore, I wanted to determine whether the *fru* gene is involved in the female assessment of individual male courtship components, rather than the integration of male courtship signals. By removing different components of male courtship (e.g. courtship song or substrate borne abdominal vibrations) or female ability to perceive courtship cues by removing the female's antennal

segments and aristae, I was able to determine that *fru* is not acting through any of these individual courtship components (Chapter 2). This does not eliminate the possibility that *fruitless* might be acting via a different sensory modality that I was not able to test Likewise; it does not preclude that the gene might be influencing female preference through the integration of multiple modalities. Future experiments should test other sensory modalities such as cuticular hydrocarbons (CHCs). These CHCs appear to serve as repellent signals to prevent interspecific mating, and not as an attractant to increase intraspecific courtship and copulation (Dweck et al., 2015). This is certainly the case in *D. melanogaster*/*D. simulans* pairs, where *D. simulans* males normally have low levels of courtship towards *D. melanogaster* females, but vigorously court oenocyteless (do not produce CHCs) *D. melanogaster* females (Billeter et al., 2009). This vigorous courtship by *D. simulans* males is abolished when the *D. melanogaster*-specific CHC compound 7,11-heptacosadiene is added to the oenocyteless *D. melanogaster* females. This is a clear indication of the role this CHC has as a repellent to *D. simulans* males, reducing their levels of interspecific courtship. It is possible that *fru* is involved in the processing of CHCs that contribute to female discrimination against interspecific matings and the potential role of olfaction in the context of *fruitless* and species-specific female preference needs to be explored further. Alternatively, *fru* might be acting through the integration of multiple sensory modalities. Therefore, identifying potential brain regions and neurons involved in the processing of mating signals and testing them for their role in species-specific female preference would be instrumental in understanding the mechanism of how *fru* is affecting species-specific female preference.

5.5 Development of tools to characterize the genetic and neural basis for species-specific female preference

There is an abundance of genetic and molecular tools available for *D. melanogaster*, but the same cannot be said of other *Drosophila* species. This can be a limitation for finding candidate genes for behavioural isolation, since *D. melanogaster* does not produce fertile hybrids with any of the other species in the *D. melanogaster* species complex, the traditional genetic mapping techniques of recombination mapping is not feasible as an approach for identifying inter-species isolation loci. Although these limitations were

circumvented by using deficiency mapping (Laturney and Moehring, 2012b), leading to the discovery of potential candidate genes like *fruitless* (Chapter 2), the lack of basic genetic tools such as *Balancer* chromosomes, in *D. simulans* impedes the ability to maintain, track or visually screen for transgenic manipulations in this species. This in turn limits the possibility of creating other useful genetic tools such as the *GAL4/UAS* system or a comprehensive gene disruption libraries.

RNA interference is a commonly used technique for silencing gene expression in *D. melanogaster* and there many resources available for targeted knockdown of specific genes in *D. melanogaster*. The genetic tools available for RNA interference in *D. melanogaster* can also be used to target only the *D. simulans* copy of a gene or transcript in *D. melanogaster*/*D. simulans* F1 hybrids. The *UAS-RNAi* transgenes targeting the *D. simulans* allele of the gene of interest can be integrated into the *D. melanogaster* genetic background, causing only the *D. simulans* allele to be silenced in F1 hybrids. This allele-specific knockdown can help target disruption of *D. simulans* genes by taking advantage of the transgenic resources available for *D. melanogaster*. This can be a viable alternative to gene editing techniques such as CRISPR that can be more difficult to achieve and maintain in species other than *D. melanogaster* due to the lack of availability of *Balancer* chromosomes in other species, a tool that facilitates generation of a disruption stock and that is necessary for the maintenance of disruptions that are homozygous lethal. In Chapter 4, I provided a detailed description of the process of designing, creating and validating short hairpin RNAi constructs for both *D. melanogaster* and *D. simulans fruitless* P2 transcripts. I was able to successfully create transgenic RNAi lines for *D. melanogaster* and *D. simulans* P2 and even though the knockdown of the transcripts did not produce a significant effect in species-specfic receptivity, it is still a very viable technique for testing the role of potential candidate genes on female preference, especially for reciprocal knockdown of the same gene in *D. simulans.* Although, allele specific RNAi can be an option for silencing gene expression in other species, there are many caveats to using RNAi, such as leaky expression, potential off-target effects, and inadequate knockdown of gene expression (Bellés, 2010; Scott et al., 2013).

By comparison, CRISPR-based genome editing is a far more effective tool for creating targeted defined deletions or gene disruptions through non homologous end joining (NHEJ), and for incorporating specific base pair changes or exogenous DNA sequences through homology directed repair (HDR) of double-stranded breaks. Either of these strategies can be very useful for testing candidate genes for behavioural isolation. Creating targeted deletions of specific exons in *D. melanogaster fru* then testing these deletions using the genetic mapping approach described in Chapter 2 can be one way to identify the role of different *fru* transcripts in species-specific female preference. Concurrently, creating reciprocal deletions of these exons in *D. simulans fru* and determining their effect can will also solidify *fruitless'* role in species-specific female preference. Finally, HDR can be employed to precisely edit the *D. melanogaster fru* P2 regulatory or coding sequence to match that of *D. simulans* and test whether *D. melanogaster* females with the *D. simulans fru* P2 sequence are less receptive to *D. melanogaster* females. This would be a major first step towards identifying the mechanism by which *fru* is influencing female preference.

5.6 Concluding remarks

The *fruitless* gene is an important regulator in male courtship behaviour, but to date there has been no evidence of the gene's role in female preference. Here, I have confirmed that *fruitless* does play a role female discrimination of interspecific males, via the non-sex specific P2 transcript with a 3' fru^{C} exon. This gene is the first to be identified for female preferences underlying behavioural isolation, and also the first instance of a behavioural role for the non sex-specific *fruitless* transcripts. Although the exact mechanism of *fruitless'* effect on species-specific female preference is unclear, the identification of a causal transcript is the first step towards characterizing the genetic basis for behavioural isolation in *Drosophila.* With recent advances in genome editing and the availability of neuroanatomical tools such as genetically encoded calcium sensors, transgenic split-GAL4s and optogenetic tools, we are in the perfect landscape to investigate and broaden the understanding of mechanisms behind species-specific mate discrimination and behavioural isolation.

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Appendices

Appendix A: Chapter 2 Supplementary material

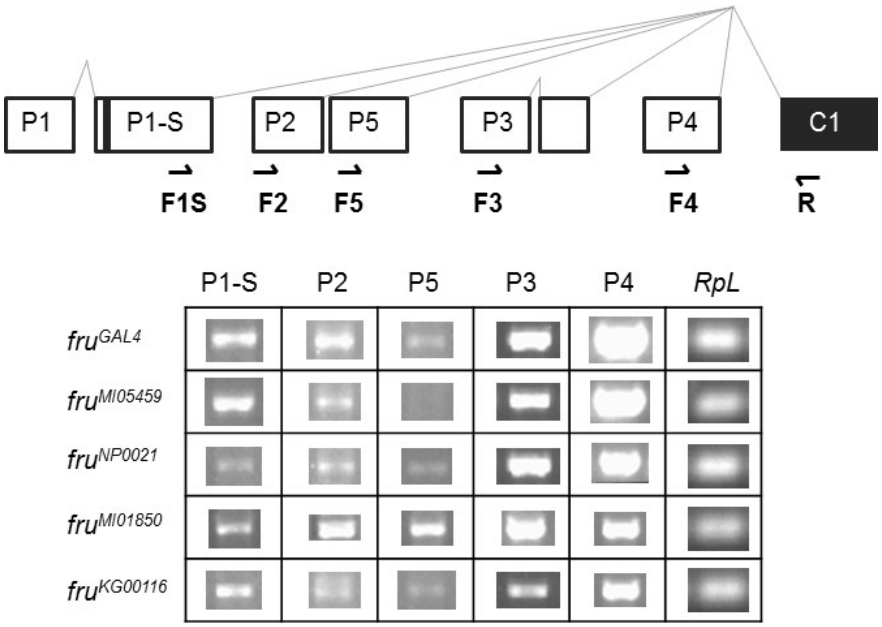

Figure S 5-1 Qualitative detection of *fruitless* transcripts using RT-PCR.
Approximate forward (F) and reverse (R) primer locations (arrows) are presented at top, aligned to the *fru* transcript (not to scale). The RT-PCR results are shown at bottom, with the gel images for the five *fru* transcripts (P1-S, P2, P5, P3, P4) and the control gene (*RpL32*). The full genotypes of the disruptions can be found in Table 1; primer sequences can be found in Table S2-1. Pure species *D. simulans* FC was also assayed with RT-PCR (data not shown), and all transcripts were present.

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Insert seq 5′:   TCCTTCTTTTTTTTTTGCTGTACGAGCCCCAACCACTATTAATTC
fru BJS seq:     TCCTTCTTTTTTTTTTGCTGTATATATTTCGACGTCTTGCCAGTC

Insert seq 3′:   AATAGTGGTTGGGGCTCGTATATTTCGACGTCTTGCCAGTCAG
fru BJS seq:     ACCTTCTTTTTTTTTTGCTGTATATATTTCGACGTCTTGCCAGTC

Rescue seq:      TCCTTCTTTTTTTTTTGCTGTATATATTTCGACGTCTTGCCAGTC
fru BJS seq:     TCCTTCTTTTTTTTTTGCTGTATATATTTCGACGTCTTGCCAGTC
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Figure S 5-2 Sequence of the *fru*^{MI05459} *Minos* insertion strain before and after the *Minos* element was rescued. The top alignments are the 5' and 3' end of the *Minos* insertion site of the intact stock, with the *Minos* element sequence highlighted in yellow. The sequences are aligned to the corresponding wild-type *D. melanogaster* sequence of *fru* (FlyBase). The bottom alignment is the same *fru*^{MI05459} strain after the *Minos* element was removed, showing a clean excision and full alignment with wild-type *fru*.

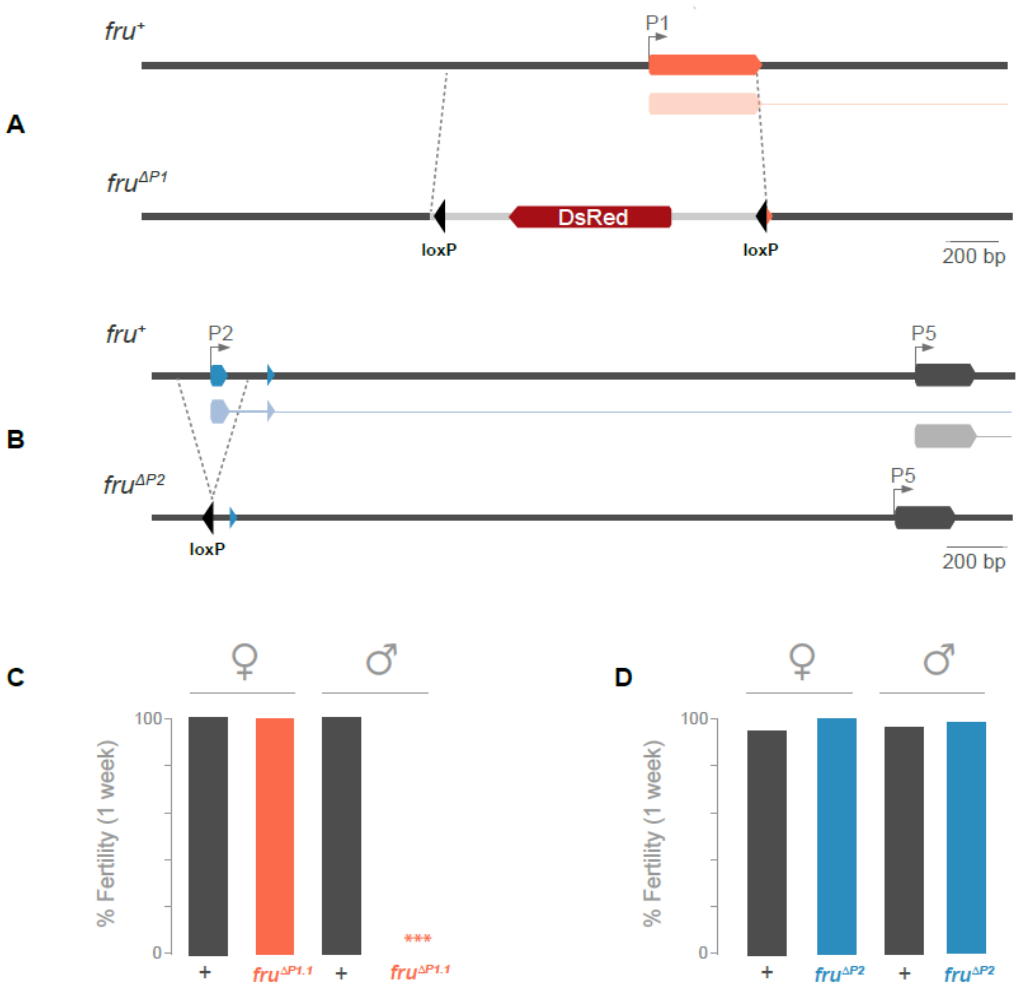

Figure S 5-3 Schematic depicting the generation of *fruitless* promoter-specific deletions targeting P1 and P2. (A) The P1 promoter was replaced by DsRed sequences flanked by *loxP* recombination sites using CRISPR-Cas9 genome editing, resulting in the P1 deletion line $fru^{\Delta P1}$ (B) The P2 promoter was removed using ends-out homologous recombination, leaving a *loxP* recombination site, resulting in the P2 deletion line $fru^{\Delta P2}$. Potential RNA transcripts produced from promoters P1, P2 and P5 are shown in light orange, blue and grey, respectively. Percent fertility of homozygous *fruitless* mutant $fru^{\Delta P1.1}$ (C) and $fru^{\Delta P2}$ (D) males and females. Statistical comparisons of *fruitless* mutant genotypes were made against *Cantons-S* (+) controls; ***$p < 0.0001$ by Fisher exact test, ($n = 30$).

Table S 5-1 List of Forward (F) and reverse (R) Primer sequences for RT-PCR and for sequencing of *Minos* element removal.

Assay	Primer name	Sequence (5' - 3')
RT-PCR	P1-S F	TCAATCAACACTCAACCCGA
	P2 F	AATCGTCGCGGTCATAAAAT
	P5 F	ACATAGACAGTGCCTCCTG
	P3 F	TCATCAGCAAATGCCTCGT
	P4 F	CCAAAAACTAAGCCCGTCAA
	COM R	AGTCGGAGCGGTAGTTCAGA
	RpL32 F	GGCATCAGATACTGTCCCTTG
	RpL32 R	CCAGTCGGATCGATATGCTAA
Minos	fruMI05459 F	AATTTCAACCGCATCTGGAC
	fruMI05459 R	ACTCATGTGGAGCCGACTTT
	fruMI05459ORI5'F	GACGAAATGCATTGGTATGTG
	fruMI05459ORI5'R	GACGAAATGCATTGGTATGTG

Table S 5-2 Primer sequences and gRNA target regions for targeted removal of fru P1 and P2 exons.

Constructs	Primer/Target Region	Sequence (5' - 3')
fruΔP1	G1_5f	GCATTTAGAATAAATTTTGTGTCGCCCTTGAACTCGATTGACGGAAGAGCCTGATTACGCTTCATTGCTGGC
	G1_5r	GAGCACTAGTAAAGATCTCCATGCATAAGGCGCGCCTAGGCCTTCTGCAGCTGCCCCTACGGGAATGACAACC
	G1_3f	GCACTACGATCGCAGGTGTGCATATGTCCGCGGCCTCTGCTGAGTGCAT
	G1_3r	GCTGAAGCAGGTGGAATTCTTGCATGCTAGCAGATGTAAGAGTACTCAAATATATTATTGAATATAAATT
fruΔP2	P2_5f	CGTACGCTGCATCTTTGACTTGCTGCGCCAAA
	P2_5r	GGCGCGCCAAATAGTAAAAAAATGCACCGGCC
	P2_3f	GGTACCAGTGAGTTAGTGCACAGATTAC
	P2_3r	AGGATCGGAAAAATCGAGAGATGCGGCCGC
gRNA Target Region	Fru5_1	TCATTCCCGTAGGGGCAATGAGG
	Fru3_3	TGCACTCAGCAGAGGCTGACTGG

Appendix B: Chapter 4 Supplementary material

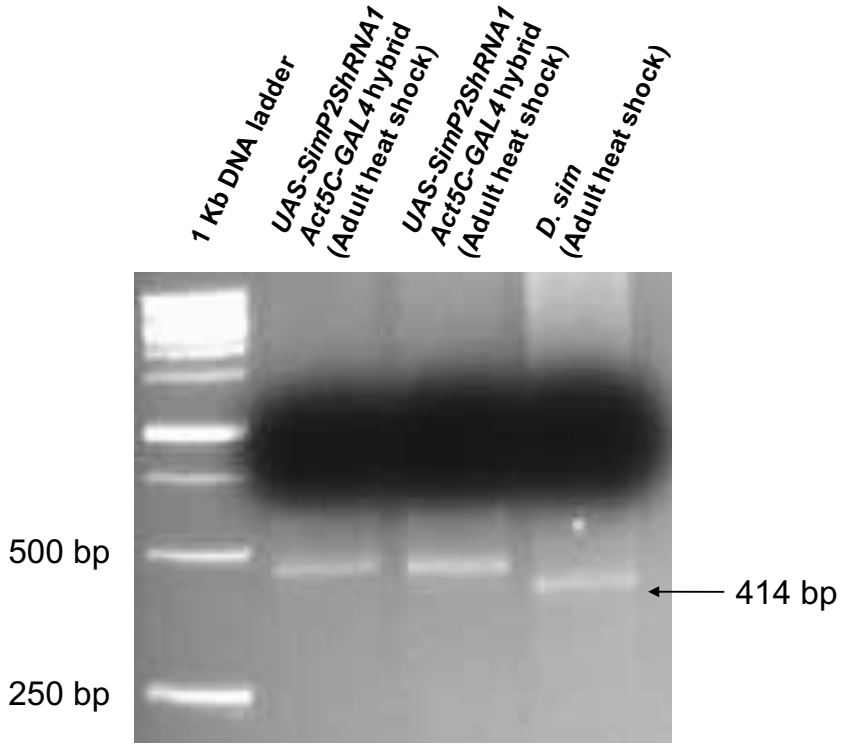

Figure S 5-4 BseY1 digestion of P2 pcr product in *Act5C-GAL4:UAS-SimP2shRNA* hybrids. Digestion of the 464bp *D. simulans* P2 PCR product is expected to produce two bands (414 bp and 50 bp). The 414 bp digested pcr product is present for wild-type *D. simulans* females, but not in the hybrids.

Table S 5-3 Forward (F) and reverse (R) Primer sequences for RT-PCR and screening for pVALIUM 20 insertion.

Assay	Primer name	Sequence (5' - 3')
RT-PCR	P2 F	AATCGTCGCGGTCATAAAAT
	P5 F	ACATAGACAGTGCCTCCTG
	P3 F	TCATCAGCAAATGCCTCGT
	P4 F	CCAAAAACTAAGCCCGTCAA
	COM R	AGTCGGAGCGGTAGTTCAGA
	RpL32 F	GGCATCAGATACTGTCCCTTG
	RpL32 R	CCAGTCGGATCGATATGCTAA
	pVAL F	ACCAGCAACCAAGTAAATCAAC
	pVAL R	TAATCGTGTGTGATGCCTACC

Curriculum Vitae

Name: Tabashir Chowdhury

Post-secondary Education and Degrees:
York University
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Honours and Awards:
Annual *Drosophila* Research Conference travel award (2017)

Related Work Experience:
Graduate Teaching Assistant
The University of Western Ontario
2013-2019

Publications:

Chowdhury, T., Calhoun, R., Neville, M., Bruch, K., Goodwin, S., and A.J. Moehring. (*revision requested*). The *fruitless* gene affects female receptivity and species isolation**.** PLoS Biology.

Dhillon, A., Chowdhury, T., and A.J. Moehring. (*revision requested*). Reproductive consequences of an extra long-term sperm storage organ. BMC Evolutionary Biology.

Oral Presentations:
Annual *Drosophila* Research Conference (2017)
Presentation title: *"not my type"* candidate gene for behavioural isolation in *Drosophila.*
San Diego, California, USA.

Synthetic Biology Symposium (2016)
Presentation title: Genetic basis of species-specific female mate preference in *Drosophila*.
London, Ontario, Canada.

Ontario Ecology, Ethology, and Evolution Colloquium (2013)
Presentation title: Egg protein variabillity in *Apis mellifera*.
London, Ontario, Canada.