THE ROLE OF MICRORNAS IN *DROSOPHILA MELANOGASTER* MALE COURTSHIP BEHAVIOR

A Dissertation

by

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ABSTRACT

Drosophila melanogaster courtship, although stereotypical, continually changes based on cues received from the courtship subject. Such adaptive responses are mediated via rapid and widespread transcriptomic reprogramming, a characteristic now widely attributed to microRNAs (miRNAs) along with other players. Here, we conducted a large-scale miRNA knockout screen to identify miRNAs that affect various parameters of male courtship behavior. Apart from identifying miRNAs that impact male-female courtship, we observed that *miR-957* mutants performed significantly increased male-male courtship and chaining behavior whereby groups of males court one another. We tested the effect of *miR-957* reduction in specific neuronal cell clusters, identifying *miR-957* activity in Doublesex (DSX)-expressing and mushroom body clusters as an important regulator of male-male courtship interactions. We further characterized the behavior of *miR-957* mutants and found that these males court male subjects rigorously but do not elicit courtship. Moreover, they fail to lower courtship efforts towards females with higher levels of anti-aphrodisiac pheromones. At the level of individual pheromones, *miR-957* KO males show a reduced inhibitory response to both 7-Tricosene (7-T) and *cis*-vaccenyl acetate (cVA), with the effect being more pronounced in case of 7-T. Overall our results indicate that single miRNAs can contribute to regulation of complex behaviors, including detection of chemicals that control important survival strategies such as chemical mate-guarding and maintenance of sex and species-specific

courtship barriers.

I also discuss the results of experiments conducted to understand whether fat body expressed miRNAs contribute to courtship regulation and whether fat body secretes miRNAs into circulation. These experiments were conducted in an effort to track in vivo inter-organ exchange of miRNAs. In the last section of this dissertation I discuss the progress made in this context and also the limitations of this approach.

DEDICATION

To Abu, Amma and Taimur who laid a strong *foundation f*or my ambitious academic journey,

To Ginger Carney, for being my *pillar* of support through her constant encouragement,

And

To Saad Abdul Aleem, for *sheltering* me from the fear of failure through his unwavering love.

Thank you all, for *building my dream* with me.

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NOMENCLATURE

CI	Courtship Index; the amount of time spent courting as a proportion of the total observation time.
Gal4	Yeast transcription factor
UAS	Upstream activating sequence, binding site for GAL4 protein
CS	Canton S, a wildtype *Drosophila melanogaster* strain
cVA	*Cis*-vaccenyl acetate; a Drosophila pheromone, aversive for *D. melanogaster* males
7-T	7-Tricosene; pheromone, inhibitory in nature for *D. melanogaster* males

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1.1 Introduction to microRNAs (miRNAs)

1.1.1 Discovery of miRNAs

miRNAs are small regulatory RNAs that range in size from ~19-24 nucleotides (nts) and exert post-transcriptional effects on gene expression via complementary base pairing with target messenger RNAs (mRNAs) by either blocking translation or decreasing message stability in their roles as expression modulators. The discovery of the first miRNA came from the characterization of the heterochronic gene *lin-4* in *Caenorhabditis elegans (C. elegans)* (CHALFIE *et al.* 1981), when it was found that *lin-4* did not encode a protein product but post-transcriptionally repressed *lin-14*, potentially by binding to complementary sequences in the 3'untranslated region (UTR) of the *lin-14* mRNA (LEE *et al.* 1993; WIGHTMAN *et al.* 1993). As interesting as this serendipitous finding was, it was an anomaly to many and was considered a unique case, relevant in nematodes only, for *lin-4* is not conserved beyond the *Caenorhabditis* genus (PASQUINELLI *et al.* 2000). It took nearly another decade after the discovery of *lin-4* RNA-mediated regulation of *lin-14* for the scientific community to recognize that there were evolutionarily conserved instances of translational repression by endogenous small

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* Parts of this section are reprinted with permission from "The in vivo genetic toolkit for studying expression and functions of *Drosophila melanogaster* microRNAs" by Hina Iftikhar, Janna N. Schultzhaus, Chloe J. Bennett, and Ginger E. Carney, 2016. *RNA Biology*, VOL. 14, NO. 2, 179–187, Copyright 2016 by Taylor and Francis Group, LLC.

small RNAs via antisense mechanisms. The major breakthrough came with the discovery of *let-7,* another non-coding RNA (REINHART *et al.* 2000) that was highly conserved across multiple species, including humans, zebrafish and fruit flies. These discoveries paved the way for investigating endogenous small RNA-mediated developmental control across animal taxa, and since then progress has been made by several groups in understanding the biogenesis pathways for miRNAs (HA AND KIM 2014) as well as their targeting mechanisms (HAUSSER AND ZAVOLAN 2014). Toward these efforts, *D. melanogaster* has proven to be one of the most amenable animal models for both in vitro and in vivo studies of miRNA biogenesis, expression and activity. The recent availability of a variety of genetic and molecular tools for manipulating *D. melanogaster* miRNA levels in vivo has greatly facilitated use of this animal model for miRNA functional analysis.

1.1.2 Biogenesis and targeting

Analysis of the *let-7* gene sequence shed light on the biogenesis pathway of miRNAs. The initial product of miRNA transcription was proposed to be the primary miRNA (pri-miRNA) transcript, which has a stem-loop structure and may contain one or more hairpins (PASQUINELLI *et al.* 2000). Later studies revealed that this pri-miRNA is processed further to yield the functional regulatory miRNA (HUTVAGNER *et al.* 2001).

miRNA genes are usually transcribed by RNA pol II to produce long primary transcripts (pri-miRNA) that are later trimmed to produce ~ 70 nt precursor miRNAs

(pre-miRNA) (LEE *et al.* 2002; LEE *et al.* 2003). However, transcription of miRNAs present within Alu repeats and other repetitive elements is carried out by RNA pol III (BORCHERT *et al.* 2006). Processing of the pri-miRNA is initiated by the RNase III enzyme Drosha, which interacts with the pri-miRNA as a part of the nuclear microprocessor complex (LEE *et al.* 2003). DGCR8, the DiGeorge syndrome critical region 8 protein (Pasha in Drosophila) interacts with Drosha in the microprocessor complex to mediate this cleavage (LANDTHALER *et al.* 2004). DGCR8 is associated with recognition of the cleavage sites (LANDTHALER *et al.* 2004) whereas Drosha forms the main catalytic component of the complex and cleaves the pri-miRNA two helical turns down from the stem loop. The precise cleavage may vary slightly based on the stem structure and possibly the sequence of the stem (ZENG *et al.* 2005). Drosha cleavage results in a pre-miRNA that has a 2 nt overhang at its 3'end (LEE *et al.* 2003). The overhang is used by the Exportin 5 protein to recognize the pre-miRNA as its substrate and transport it out of the nucleus (Figure 1) (YI *et al.* 2003). The enzyme Dicer cleaves the pre-miRNA via its RNase III domain separating the stem from the loop of the hairpin. The cleavage is strictly ATP dependent and gives the mature miRNAs their characteristic terminal structures (2 nt overhang and hydroxyl group at the 3' end). Interestingly Dicer, the enzyme involved in the latter step of miRNA processing was discovered before Drosha, the enzyme needed for the first step. Hutvagner et al. (2001) showed that reducing Dicer resulted in the accumulation of *let-7* precursor miRNAs in HeLa cells. On the other hand, Drosha was shown to cleave pri-miRNA into pre-miRNA

in vitro, and reducing Drosha expression via RNAi resulted in the accumulation of pri-miRNA (LEE *et al.* 2003).

After processing by Dicer, the RNA duplex is loaded onto the RISC loading complex. In mammals, there is a single Dicer protein. Dicer mediates loading via formation of heterodimers of Dicer and its associated RNA binding proteins such as TRBP (trans-activation response RNA binding protein) (CHENDRIMADA *et al.* 2005) and PACT (protein activator of PKR) (LEE *et al.* 2013). Hsc70/Hsp90 chaperone proteins also facilitate the loading. It is suspected that miRNAs can associate with any of the AGO proteins (through 1-4) indiscriminate of their sequence. It is clear that siRNA mediated cleavage requires AGO2 in mammals, but it is not clear if one of the four AGO proteins preferentially associates with miRNAs since miRNA complexes with all four AGO proteins have the capacity to translationally repress the miRNA target. However, only AGO2 has Slicer activity (SU *et al.* 2009). It has been speculated that AGO protein contribution to miRNA-mediated silencing correlates with their expression levels, thus making the most abundant AGO2 the most relevant (WANG *et al.* 2012a). However, a few recent studies have opened up the possibility that the distribution of AGO for miRNA silencing is coordinated by additional mechanisms, such as action of LIM-domain-containing proteins, rather than just expression differences (BRIDGE *et al.* 2017; FU *et al.* 2017).

The Ago protein uses its N-terminal domain to unwind the duplex and incorporate only one of the strands of the duplex (KWAK AND TOMARI 2012). This is the guide strand and it is either the 5' or the 3' strand of the duplex. The complementary

strand, known as the passenger strand, is thought to be degraded in most cases (KIM 2005). However, there are exceptions, such as mir376a*, which is retained as a mature miRNA product and not degraded by default (CHOUDHURY *et al.* 2012). The passenger strand, in several cases, has been shown to mediate gene regulation and often resides in AGO1 complexes (OKAMURA *et al.* 2004). Strand selection is dependent on the asymmetric thermodynamic stability of the RNA duplex. Statistical analyses and thermodynamic profiling have showed that the strand with lower stability at the 5' end of the pre-miRNA hairpin is incorporated into the RISC complex (KHVOROVA *et al.* 2003).

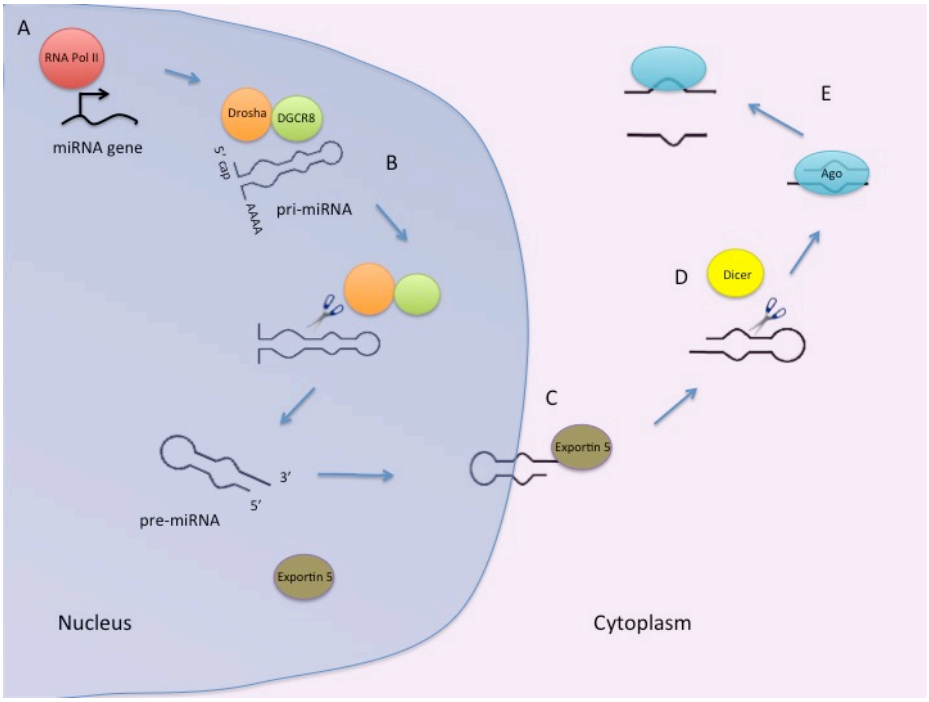

Figure 1. Canonical pathway for the biogenesis of miRNAs A) miRNA genes are predominantly transcribed by RNA Pol II, exceptions include miRNAs within ALU repeats and other repetitive elements. B) The transcription product, known as the primary miRNA (pri-miRNA) is recognized and cleaved by the Drosha/DGCR8 complex to release the shorter precursor miRNA (pre-miRNA) C) pre-miRNA is transported out of the nucleus by the Exportin V protein. D and E) Dicer cleaves off the stem loop from pre-miRNA to release the double-stranded product from which only one strand will be incorporated into the RISC.

1.1.3 Regulation of gene expression

The RISC complex targets the mature miRNA to the 3'UTR of its target mRNA. The binding is determined by multiple factors, primarily by the base pairing of the 'seed

region' (~6-8 nts) at the 5' end of the miRNA. Nucleotides 2-7 of the miRNA correspond to the seed region. Most canonical target sites are defined as sites that have a nucleotide complementary to nucleotide 8 of the miRNA in addition to matching the seed region, and/or they have an adenine opposite position 1 of the miRNA (BARTEL 2009; HAUSSER AND ZAVOLAN 2014). Grimson et al. (2007) uncovered five main contextual features of miRNA targets that enhance the efficacy of miRNA targeting. One of these was the proximity of target sites for miRNAs that are co-expressed. Homologous binding of the miRNA targets to positions 12-17 of the miRNAs, more importantly the nucleotides 13-16, also improved targeting. It was also shown that functional target sites were mostly present in the vicinity of an AU rich region. Furthermore, the distance of the target site from the stop codon also influences the efficacy of repression. Most conserved sites were found ~15 nts away from the stop codon. Additionally, sites that were near the ends of the 3'UTR were more effective than the ones in the center (GRIMSON *et al.* 2007).

The RISC complex can prevent the translation of the miRNA targets in several ways. The initiation of translation can be inhibited by recruiting proteins which impact the stability of the target mRNA such as deadenylase, decapping enzymes, 3′ and 5′ exonucleases, and endonucleases (VALENCIA-SANCHEZ *et al*. 2006; FABIAN *et al*. 2010). Apart from recruiting these proteins, RISC can also compete with cap binding proteins or translation initiation factors. These proteins are needed for the circularization of mRNA, a step required for the initiation of translation (MATHONNET *et al*. 2007). RISC can also inhibit translation post-initiation by interfering with ribosomal subunits or

elongation factors (FABIAN *et al*. 2010). Apart from translational inhibition, miRNAs can also lead to degradation of their targets by recruiting them to P-bodies (LIU *et al*. 2005).

The read-out of such interactions is typically believed to be a reduction in mRNA and protein levels but, as discussed in a recent review (HAUSSER AND ZAVOLAN 2014), the magnitude of repression by a single miRNA is generally low (rarely higher than 2-3 fold), and there can be other consequences of miRNA-target interactions. miRNAs and transcription factors can form feed forward loops to counteract leaky transcription and hence reduce the cell-to-cell variability in the levels of miRNA targets (HAUSSER AND ZAVOLAN 2014). miRNAs that influence their targets depending on the concentration of targets (at higher rate of transcription the targets can escape miRNA mediated degradation) can help set the threshold for transcriptional activity that needs to be passed for target accumulation. Targets such as certain pseudogenes and circular RNAs also seem to function to sequester the miRNAs (HAUSSER AND ZAVOLAN 2014). Apart from translational repression, cases have also emerged where miRNAs have switched from repressing to upregulating the translation of their targets based on the physiological context (VASUDEVAN *et al.* 2007).

1.1.4 Phenotypic effects

Currently, miRBase reports as many as 2588 annotated human mature miRNAs and it has been suggested that over 60% of the human protein-coding genes have conserved target sites for miRNAs (FRIEDMAN *et al.* 2009). Such abundance of miRNAs with potentially widespread targeting indicates that most biological processes and phenotypes

would be affected by miRNAs in some way. *lin-4* and *let-7*, for example, the miRNAs discovered earliest, were both involved in regulating developmental timing in *C. elegans* (LEE *et al.* 1993; WIGHTMAN *et al.* 1993; REINHART *et al.* 2000). Since then *let-7* has been shown to be involved in a diverse array of processes across species. In flies *let-7* is needed for neurogenesis (KAWAHARA *et al.* 2012; KUCHERENKO *et al.* 2012) as well as metamorphosis (CAYGILL AND JOHNSTON 2008; SOKOL *et al.* 2008). In humans, as well as other species such as *C. elegans, let-7* has been shown to suppress tumor growth by targeting various oncogenic pathways (WANG *et al.* 2012c), and it is needed for limb development in chicken and mice (DARNELL *et al.* 2006). Most commonly *let-7* family members are needed to promote cellular differentiation for developmental transitions and often act as tumor suppressors (ROUSH AND SLACK 2008; WANG *et al.* 2012c), reflecting a few of the many biological processes influenced by miRNAs.

miRNAs can act as buffers to prevent leaky expression or act as switches to bring about drastic changes in a cell's transcriptional profile and ultimately result in the inactivation of regulatory pathways (KLOOSTERMAN AND PLASTERK 2006). The latter is commonly observed during development, and the best known examples of such developmental switches include the above mentioned *let-7* miRNA, which downregulates a key developmental regulator in *C. elegans*, LIN-41. LIN-41 in turn mediates larval to adult transition by allowing cells to differentiate (PASQUINELLI *et al.* 2000; REINHART *et al.* 2000; ROUSH AND SLACK 2008). miRNAs also have been shown to be important for cell-cycle exit and cellular differentiation in vertebrates (HUDISH *et al.* 2013). Apart from cellular differentiation, miRNAs can be required for cell fate

determination during development. Cell lineage specification, however, appears to occur appropriately even in the absence of miRNAs (STATON AND GIRALDEZ 2011).

There are several downstream effects of such regulation including impacts on body size and organogenesis. In *Drosophila melanogaster* the miRNA *bantam* has been shown to control body size by suppressing ecdysone activity in systemic cells to promote growth (BOULAN *et al.* 2013). The expression of several other miRNAs, required for molting related developmental transitions in Drosophila, is triggered in response to changes in the levels of ecdysone and juvenile hormone (SEMPERE *et al.* 2003). Formation of individual organs and tissues requires several miRNAs in vertebrates as well. Some examples include the expression of *mir-9* and *mir-7* during teleost brain morphogenesis (BIZUAYEHU AND BABIAK 2014), the requirement of *mir-204* for medaka fish eye development (CONTE *et al.* 2010) and the dependence of mouse Islet cell genesis on the production of miRNAs (LYNN *et al.* 2007). In several cases the expression of the miRNA is also required to maintain the tissue's state after development. Numerous miRNAs are required for the development of the nervous system in Drosophila (SOREQ 2014), but even after development of the nervous system, activity dependent synaptic growth in the neuromuscular junction requires expression of several miRNAs including *mir-8* (NESLER *et al.* 2013). Similarly, *mir-1* expression is needed for the development and maintenance of fly muscle (KLOOSTERMAN AND PLASTERK 2006).

As mentioned above, the steady expression of certain miRNAs is needed for maintenance of cell states but for some miRNAs age-associated change in expression pattern is observed. Such miRNAs are commonly implicated in processes such as

neurodegeneration and metabolism (XU *et al.* 2003; LIU *et al.* 2012; ESSLINGER *et al.* 2013; NOREN HOOTEN *et al.* 2013).

A number of miRNAs have also been shown to be important for lipid metabolism. These include some that are expressed in the liver during development but are also required later to maintain the differentiated state of cells and develop other functions such as contributing to lipid metabolism. One example is *mir-122*. Inhibition of *mir-122* in mice results in lower levels of both high and low density lipoproteins in plasma (ESAU *et al.* 2006). Other examples of miRNAs that influence lipid metabolism include the *mir-33/mir33** duplex. It has been suggested that in humans, mice and nonhuman primates, both arms of the *mir-33/miR-33** duplex are involved in lipid metabolism as they inhibit enzymes vital for cholesterol and fatty acid metabolism (GOEDEKE *et al.* 2013). Other miRNAs are also differentially expressed at different stages of adipogenesis and are suggested to be potential therapeutic targets for obesity (CHEN *et al.* 2014a).

Another well-characterized phenotypic consequence of miRNAs is their role in regulating apoptosis. In Drosophila *mir-14* and *bantam* are two prominent examples of miRNAs that are involved in the control of cell death. *mir-14* has been shown to suppress Rpr, Hid, Grim, Dronc and Drice controlled apoptotic events (XU *et al.* 2003). Other miRNAs that antagonize apoptosis include *mir-278* and *mir-2*. In mammals miRNAs also seem to be involved in apoptic control, for example, *mir-15* and *mir-16* induce apoptosis by suppressing BCL2. There are cases where the same miRNAs can either induce apoptosis through oncogenic activity or act as tumor suppressors

depending on the cell type and conditions, e.g. *mir-17* (JOVANOVIC AND HENGARTNER 2006).

Many phenotypes are regulated by miRNAs that are under circadian control. *mir-219* and *mir-321* are rhythmically expressed in the mammalian suprachiasmatic nuclei (SCN) of the hypothalamus (the master circadian clock in mammals) and influence cellular excitability. While *mir-219* is under the control of clock, *mir-132* is light-inducible. *mir-219* and *mir-321* in turn influence circadian period length and clock-resetting in the SCN respectively (CHENG *et al.* 2007). In Drosophila a cluster of six miRNAs, *mir-956-964,* that is rhythmically expressed controls the timing of feeding. Interestingly, nutrition itself acts as a trigger for the expression of these mirs, showing that here miRNA mediated transcriptional control is part of a feedback loop (VODALA *et al.* 2012). Feedback loops are a recurring phenomenon as several other miRNAs have also been shown to mediate responses that, in turn, regulate the expression of the very same mirs (TSANG *et al.* 2007; OKADA *et al.* 2014).

1.1.5 Cell autonomous versus non-cell-autonomous functions

miRNAs influence processes at a cellular level where they are involved in either maintaining cell states or activating/inactivating signaling pathways. These induce developmental transitions or behavioral responses, some of which I have mentioned above. A plethora of processes are under miRNA-mediated control and these miRNAs are themselves often expressed in response to triggers such as hormones and nutrition, in other words, a combination of environmental and physiological cues.

In most cases miRNAs are assumed to function cell-autonomously. However, there are several pieces of evidence indicating that miRNAs may have non-cell-autonomous functions as well. For example, the possibility of non-cell-autonomous functions for miRNAs is suggested by their existence in wide-ranging body fluids (CHEN *et al.* 2008; WEBER *et al.* 2010; SOHEL *et al.* 2013) and transfer to recipient cells (MITTELBRUNN *et al.* 2011). In samples derived from healthy human subjects, miRNAs were identified in all 12 biofluids tested, including those that are sex-limited such as seminal and amniotic fluid and breast milk and colostrum (WEBER *et al.* 2010). miRNA concentrations varied widely across these tissues, although 61 miRNAs were common to all 12. Interestingly, fluid-specific miRNAs were detected in plasma, tears, breast milk and seminal fluid, while urine, bronchial lavage, and pleural fluid only contained miRNAs that were also present in other body fluids (WEBER *et al.* 2010). Some miRNAs are transferred to adjacent cells via direct cell-to-cell contact (Rechavi *et al.* 2009; Katakowski *et al.* 2010; Aucher *et al.* 2013), while others appear to be transported via extracellular vesicles (Valencia *et al.* 2014) or miRNA/protein complexes (Vickers *et al.* 2011; Tabet *et al.* 2014).

Although the biological importance of the miRNAs in biofluids is not clear, changes in the levels of some miRNAs are correlated with a disease state, making fluid miRNAs useful biomarkers for disease and other conditions (MITCHELL *et al.* 2008; GUPTA *et al.* 2010; REDELL *et al.* 2010).

1.2 Genetic approaches to studying miRNAs in Drosophila

In recent years there has been a dramatic increase in the number of studies that have utilized Drosophila to study the contribution of miRNAs to essential biological processes (WENG *et al.* 2013; BARRIO *et al.* 2014; BUSTO *et al.* 2015; CHEN AND ROSBASH 2017). Among the key characteristics of *D. melanogaster* that make it a tractable model for in vivo biological studies are its relatively short developmental period and lifespan, its compact genome, and the reasonable ease with which researchers can manipulate gene expression in vivo. As a result of these features, *D. melanogaster* has proven to be a useful animal model for studying a wide variety of biological processes, and researchers have developed genetic tools that allow manipulation of gene expression levels in vivo. There are a variety of strategies that can be used to generate gene-specific deletion mutations, strains are available for RNAi-mediated reduction of gene expression, and there are tools for overexpression and misexpression studies. These deletions can be stably maintained over balancer chromosomes in Drosophila. These strategies have been applied in recent years to the study of functional activities of miRNAs in specific cells and tissues in whole animals. The recent public availability of strain libraries that allow researchers to manipulate the expression levels of most Drosophila miRNAs makes it now possible for researchers to carry out large-scale screens to investigate the contributions of miRNA activity to any number of interesting biological processes.

1.2.1 Deletion mutants

One important strategy in the Drosophila geneticist's toolkit is the ability to produce miRNA-specific deletion mutants. While several labs have created strains in which one miRNA or a cluster of nearby miRNAs is deleted, a more comprehensive library of 80 targeted miRNA knockout strains covering 104 miRNA genes was created by ends-out homologous recombination (CHEN *et al.* 2014b). miRBase (http://mirbase.org, release 21), a searchable online database for miRNA sequences in 223 species, identifies 256 miRNA precursors that are cleaved to produce 466 mature miRNAs in *D. melanogaster*. The combined set of strains deletes 130 of these miRNA-encoding loci either singly or in clusters and is available at the Bloomington Drosophila Stock Center (http://fly.bio.indiana.edu/). Drosophila miRNAs expressed at very low levels or those that are not present in other taxa were not targeted for mutation because they were not expected to produce developmental phenotypes (CHEN *et al.* 2014b).

Phenotypic evaluation of the set of 130 strains revealed that few of the single miRNA deletion alleles cause lethality, indicating that they are not required for development (CHEN *et al.* 2014b). This result is not surprising given the observations that miRNAs are most often involved in fine tuning gene expression (SELBACH *et al.* 2008) and that multiple miRNAs often work in concert to affect specific mRNA targets (SELBACH *et al.* 2008; BARTEL 2009). Indeed, few individual miRNA mutants in *C. elegans* had phenotypic effects on either development or viability (MISKA *et al.* 2007), although mutant effects were enhanced in sensitized genetic backgrounds (BRENNER *et al.* 2010).

Mutant survival to adulthood provides an opportunity to examine adult phenotypes from miRNA knockout in many of these strains. Evaluation of stage-specific developmental survival and several adult phenotypes, including lifespan, hemolymph-brain barrier permeability, fertility and ovary morphology, demonstrated that greater than 80% of the miRNA deletion strains cause at least one phenotype. Generally, only the most highly expressed miRNAs produce phenotypes (BACCARINI *et al.* 2011; MULLOKANDOV *et al.* 2012), but Chen et al. identified only a few instances where phenotypic effects correlated with miRNA expression levels (CHEN *et al.* 2014b).

1.2.2 General tools for miRNA overexpression or misexpression

Beyond gene-specific loss-of-function mutations, one of the most useful tools available to Drosophila researchers studying in vivo gene activity is the GAL4/UAS system (BRAND AND PERRIMON 1993) and related strategies. The GAL4/UAS system provides researchers with the ability to manipulate and control gene expression temporally and spatially within the animal. In short, the yeast GAL4 transcriptional activator is expressed under the control of a ubiquitous or tissue-specific enhancer (Figure 2A) and can be used to activate expression of any gene that is cloned downstream of the UAS binding sites that specifically interact with the GAL4 protein. For example, it is possible to overexpress or misexpress genes ubiquitously when enhancers such as those for the *actin* and *ubiquitin* genes are used to control *gal4* expression (e.g., *actin-gal4* or *ubi-gal4*); tissue-specific enhancers can be used to limit GAL4 expression and, hence, gene-of-interest expression to desired tissues (Figure 2A). The *enhancer-gal4* and *UAS-gene-*

of-interest constructs are maintained in separate strains until an experiment is initiated, when a simple genetic cross allows production of offspring that contain both constructs (*enhancer-gal4/UAS-gene-of-interest*) for phenotypic testing.

Enhancements to this GAL4/UAS system include the ability to introduce a temperature sensitive *gal80* allele that can be used to control temporal expression of *gal4* (MCGUIRE et al. 2004). In this case, animals raised in the permissive temperature range of 19-22ºC cannot activate GAL4-mediated gene expression. Increasing the temperature to 30ºC inactivates $GAL80^{ts}$, enabling GAL4-controlled gene expression to proceed (Figure 2B). A related strategy, the GeneSwitch system, relies upon fusion of the hormone binding domain from a nuclear receptor, such as the progesterone receptor (PR), to the GAL4 DNA binding domain, thereby sequestering GAL4 in the cytoplasm. When PR is bound to the RU486 ligand, which is usually provided to the flies in a food source, the GAL4/PR/RU486 complex is transferred to the nucleus to initiate GAL4-medited gene expression in the desired location (ROMAN *et al.* 2001; MCGUIRE *et al.* 2004).

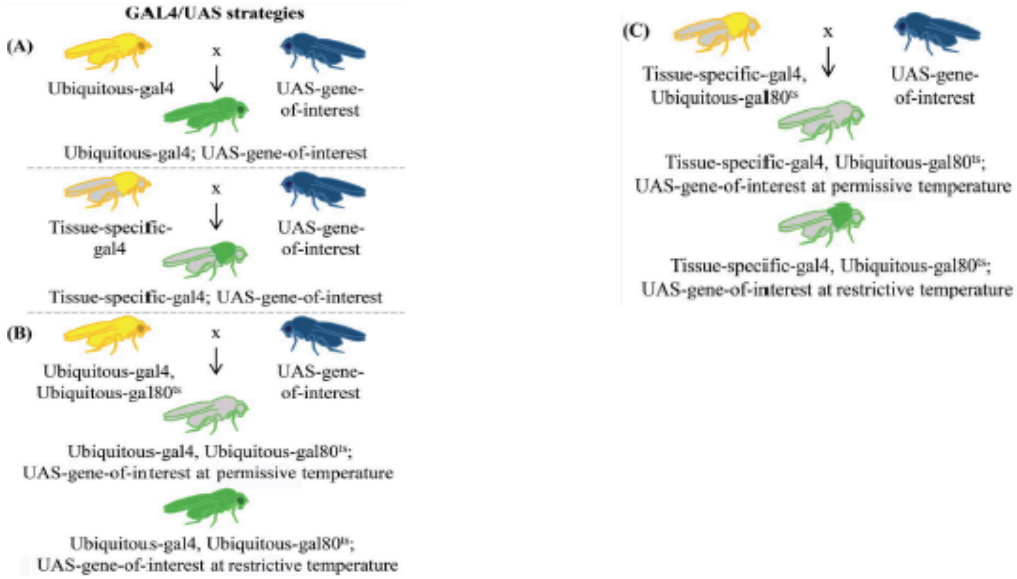

Figure 2. Variations of the GAL4/UAS system in common use (A) The GAL4/UAS system can be used to manipulate gene expression either ubiquitously or in a tissue-specific manner (illustrated in thoracic muscle as an example). Crosses between a strain carrying *enhancer-gal4* (ubiquitous or tissue-specific enhancer) and a strain with the *UAS-gene-of-interest* (gene coding sequence, RNAi, miRNA-sponge, etc.) produce flies containing both elements. In *enhancer-gal4* parents and progeny, GAL4 is always expressed based on the activity of the enhancer (represented by yellow areas). In progeny from the cross, the *UAS-gene-of-interest* will be activated where GAL4 is present, either ubiquitously or restricted to the same tissue-specific regions as the driver (represented by green in the figure). (B) Temporal control of GAL4/UAS activity can be achieved by repression of GAL4 by Gal80^{ts} which restricts activity at low temperatures (represented by grey areas) but is inactivated at higher temperatures, thereby allowing expression of the gene of interest (green body) (IFTIKHAR *et al.* 2017)

1.2.3 miRNA sponges for reducing miRNA activity

As for protein-coding genes, the GAL4/UAS system can be employed to express miRNA-encoding genes or to express RNAs that inhibit miRNA activity. Therefore, another important tool in the Drosophila arsenal for reducing miRNA activity in cells is a set of UAS-inducible strains that express miRNA "sponges". These strains produce transcripts with repetitive sequences that are complementary to a specific miRNA, with the end goal of "soaking up" miRNAs through their interaction with the sponge sequence rather than the target mRNA, hence decreasing miRNA activity in cells (LOYA *et al.* 2009; FULGA *et al.* 2015). Therefore, any observed phenotypes resemble those of reduced function or loss-of-function mutations.

There is a publicly available set of Drosophila *UAS-miRNA* sponge alleles covering 141 miRNAs, most of which are closely related to human miRNAs (FULGA *et al.* 2015). The *UAS-miRNA-sponge* (*UAS-miRNA-SP*) constructs consist of 20 copies of the appropriate miRNA complimentary sequence cloned into the 3' untranslated sequence of mCherry. Expression of mCherry allows visualization of the sponge expression pattern. The constructs were integrated at the attP40 (2nd chromosome) and attP2 (3rd chromosome) sites to reduce positional effects and equalize expression, yielding 282 independent strains. Comparisons of effects of miRNA null mutations (CHEN *et al.* 2014b) to those from globally expressed *UAS-miRNA-SP* revealed general concordance between the null and miRNA sponge phenotypes, and miRNA sponge effects were dose dependent with strains containing identical sponge insertions on both chromosomes producing stronger effects (FULGA *et al.* 2015). Similarly to analysis of

knockout mutation (CHEN *et al.* 2014b), experiments using the miRNA sponges did not reveal strong correlations between miRNA expression levels and phenotypic effects from miRNA-sponge expression (FULGA *et al.* 2015).

Such miRNA-sponge strains are advantageous from the perspective that they can be used as a means for validating null mutant phenotypes as well as to probe tissue-specific and stage-specific activities of individual miRNAs or groups of miRNAs. For example, adult-specific reduction of miRNA activity can be achieved with the GAL80^{ts} system (Figure 2B), and the phenotypic effects can be compared to those that occur when the same miRNA is completely deleted. miRNA sensor strains allow researchers to monitor the effects of *UAS-miRNA-SP* or *UAS-miRNA* misexpression or overexpression (described below) through their effects on target gene 3'UTRs. These constructs function by expressing a cell autonomous marker sequence, such as GFP, fused to a 3'UTR derived from a gene known to be regulated by a specific miRNA. Successful miRNA-mediated inhibition is indicated by a decrease in GFP expression (BEJARANO *et al.* 2012; FULGA *et al.* 2015). Currently, sensors are not available for all miRNAs, and many more will need to be developed to meet the needs of specific investigators. Moving forward, it also will be important for additional sponge strains to be created that are specific to the remaining miRNAs so their functions can be determined.

1.2.4 miRNA tools for overexpression or misexpression

Several groups have created large-scale libraries of *UAS-miRNA* strains that allow over- and misexpression of miRNAs as a complementary strategy to analyzing miRNA loss-

of-function or reduced function effects (BEJARANO *et al.* 2012; SCHERTEL *et al.* 2012; SZUPLEWSKI *et al.* 2012). Such controlled miRNA expression has several applications. It can be used to rescue knockout mutants by expressing the miRNAs in deletion backgrounds, providing a means to verify that phenotypes identified from knockout mutations are due to deletion of the miRNA in question. While researchers often employ a globally expressed *enhancer-gal4*, such as *tubulin-gal4* or *actin-gal4*, for rescue experiments, selection of spatially limited *enhancer-gal4* constructs allows researchers to determine in which tissues the miRNA activity is required to restore the phenotype. The *UAS-miRNA* strains also can be used to overexpress or misexpress miRNAs in a wild-type genetic background, either throughout the animal or in selected locations, to uncover phenotypic effects from gain-of-function activity, lending insights into the various processes that miRNAs regulate. This approach is a particularly powerful for assigning functionality to miRNAs that do not have obvious phenotypes in loss-of-function or knockdown screens.

One research group generated strains to express 149 distinct miRNA hairpins either singly or as co-expressed clusters, and the researchers produced multiple insertion strains for each *UAS-miRNA* to create an allelic series (BEJARANO *et al.* 2012). These strains have an added tag, either Ds-Red or luciferase, that allows one to verify the location of *UAS-miRNA* expression in a cell autonomous manner. *UAS-Ds-Red-miRNA* constructs were randomly integrated into the genome via P-element transgenesis, while the *UAS-luc-miRNA* constructs were non-randomly inserted at the attP2 docking site at position 68A4 on the 3rd chromosome (BEJARANO *et al.* 2012). While expression of the

P-element-based *UAS-miRNA* may be affected by insertion location, the 107 *UAS-luc-miRNA* insertions in the attP2 landing site are less likely to suffer from positional effects on expression. Ubiquitous overexpression of approximately two-thirds of the *UAS-miRNA* transgenes resulted in embryonic or larval lethality, while targeted overexpression in wing discs phenocopied effects of mutations in known wing developmental pathways, providing a starting point for investigating how specific miRNAs function in signaling pathways via their regulatory effects on mRNAs (BEJARANO *et al.* 2012).

A second library covers 180 evolutionarily conserved and highly expressed miRNAs (SCHERTEL *et al.* 2012). All of the *UAS-miRNA* constructs in this group were integrated at the attP landing site at position 86Fb on the 3rd chromosome to reduce positional effects on expression. Overexpression caused developmental or observable phenotypes in wings or eyes for 78/180 (43%) of the insertions.

The third set of strains allows overexpression of 89 miRNAs or clusters for a total of 109 miRNAs (SZUPLEWSKI *et al.* 2012). Most *UAS-miRNA* constructs were inserted at defined sites, but a few (marked with Ds-Red) are from random insertions in the genome. A unique feature of this set of strains is that some of the miRNA sequences were cloned into UAST-based vectors for somatic expression, while vectors with a UASP backbone were used to allow germline overexpression of miRNAs. The strains from all three research groups are available at public Drosophila stock centers.

Fortunately, the miRNA strains from labs that have created strain libraries are often in a similar genetic background (BEJARANO *et al.* 2012, Chen *et al.* 2014b, FULGA

et al. 2015, SCHERTEL *et al.* 2012, SZUPLEWSKI *et al.* 2012). For *UAS-miRNA-SP* alleles, comparisons also can be made to a "scrambled" control, which randomizes the sequence of the miRNA under study (FULGA *et al.* 2015. However, "scrambled" controls are available only a small number of *Drosophila* miRNAs and have been produced on an as-needed basis by individual labs, severely limiting the current utility of this approach. Therefore, once miRNAs of interest are identified from initial screens, the alleles can be backcrossed into a common background for further analysis or the effects can be validated using other genetic and molecular approaches.

miRNA overexpression is also being used not only to explore the effects of the miRNAs themselves but also to obtain down regulated gene targets for follow-up screens using mRNA-specific RNAi (BHAT AND JONES 2016; YOU *et al.* 2018)

1.3 miRNAs and the study of behavior

The availability of sophisticated genetic tools in *Drosophila melanogaster,* has shed light on new roles for clustered as well as individual miRNAs in regulation of complex phenotypes (BEJARANO *et al.* 2012; SCHERTEL *et al.* 2012; CHEN *et al.* 2014b; FULGA *et al.* 2015). The effects of miRNA manipulation in Drosophila are perhaps more pronounced than several other model organisms because they are often studied with very high spatial and temporal specificity (BUSTO *et al.* 2015; YOU *et al.* 2018). Many of the Drosophila miRNA candidates for regulation of complex phenotypes such as behavior or immune responses have emerged in the past 5 years from large-scale loss-of-function or misexpression screens (BHAT AND JONES 2016; ATILANO *et al.* 2017; KIM *et al.* 2017; LI

et al. 2017). Initial screens in these studies typically show that a significant proportion of the miRNAs examined influence the phenotypes tested, but few candidates have been successfully validated by additional approaches. Interestingly, the effects observed with validated targets are often post-developmental (as elucidated by restricting expression in adults) and not necessarily correlated with miRNA abundance in the whole organism (VODALA *et al.* 2012; KIM *et al.* 2017).

Screens conducted with miRNA knockout or over-expression lines have improved our understanding of miRNA contributions to behaviors such as larval mobility (PICAO-OSORIO *et al.* 2015), olfactory learning (LI *et al.* 2013), circadian behavior (YOU *et al.* 2018), female receptivity to male courtship (FRICKE *et al.* 2014), feeding and foraging behavior (KIM *et al.* 2017), and even sleeping (GOODWIN *et al.* 2018). Behavioral modulation can be achieved by miRNAs in several ways. miRNAs control neuronal differentiation and morphology and could hence be required for the formation of specific neuronal circuits (MCNEILL AND VAN VACTOR 2012). In some cases the activity of specific neuronal clusters is impacted such as in the case of larval self-righting behavior where *miR-iab4* mediated targeting of *ultrabithorax* is critical for correct firing of neurons involved in the behavior (PICAO-OSORIO *et al.* 2015).

The roles of miRNAs in control of motor behaviors have also been reported for more complex organisms. For example, *miR-182* has been shown to regulate motor control in mice. *miR-128* deficiency in Dopamine Receptor 1-expressing neurons leads to hyperactivity in young mice (TAN *et al*. 2013). Loss of *miR-124* has also been associated with neurological disorders. One recent report identified AMPA Receptor

subunits as the targets of *miR-124* in mice that displayed deficits in social behaviors (GASCON *et al*. 2014).

1.4 *Drosophila melanogaster* male courtship behavior as a model

miRNA contribution to several behaviors including olfactory learning and feeding has now been demonstrated using *Drosophila melanogaster* as a model system. Another robust Drosophila behavior is male courtship behavior (LI *et al.* 2013) , which provides an excellent behavioral model for a number of reasons. For example, the steps comprising the behavior are very well-characterized and easy to score in lab. Most importantly courtship relies on a number of sensory inputs including visual and olfactory cues. Furthermore, the sex-determination hierarchy of genes underlying the behavior is also very well understood (HALL 1994; TAYLOR *et al.* 1994; YAMAMOTO *et al.* 1998). Courtship behavior is innate yet constantly modified in response to environmental stimuli. In fact, males demonstrate associative and non-associative learning in response to the feedback received from the courtship subject. Therefore, courtship behavior also provides an interesting paradigm for studies aimed at understanding behavioral plasticity and decision making (GRIFFITH AND EJIMA 2009).

Male courtship behavior is initiated when a male receives visual and olfactory cues (in the form of volatile pheromones) from the female subject. The male then orients himself towards the female and taps her with his forelegs (which have gustatory receptors) to taste the pheromones present on the female's cuticle. This step is followed by the male chasing the female and 'singing' while he does so. Singing involves

unilateral extension and vibration of the wing. The vibration frequency of the wing is species-specific. The male also attempts to lick the female genitalia and eventually attempts to copulate with the female when she is receptive and bends her ovipositor enough to allow the male to mount her (HALL 1994).

Drosophila courtship is a behavior essential for the survival of the animal. It is therefore buffered by multiple layers of regulatory checks and balances. Several studies have uncovered the major genes that regulate this behavior, but a lot remains unanswered about mechanisms that ensure tight spatial and temporal regulation of these genes.

1.5 Dissertation objectives

The aim of this dissertation is to identify miRNAs that contribute to regulation of male courtship behavior in Drosophila. Chapter 2 describes a screen carried out with miRNA knockout mutant males to identify candidates that cause aberrations in various parameters of male courtship. I present further analysis of two of the validated candidates (*miR-263b* and *miR-317*). Chapter 3 describes the behavioral characterization of *miR-957* mutant males that display inter-male courtship. Chapter 4 discusses my efforts to understand if fat body expressed miRNAs are important for Drosophila male courtship behavior. I show that reducing miRNA biogenesis in the fat body lowers courtship efforts by males. I provide data that suggests the fat body secretes miRNAs and discuss potential ways to further understand this phenomenon.

2. GENETIC SCREEN TO IDENTIFY miRNAs INVOLVED IN DROSOPHILA MALE COURTSHIP BEHAVIOR

2.1 Introduction

We understand today that innate behaviors, essential for an animal's survival and reproductive success, are often controlled by genes that operate in robust neural circuits (DE BONO AND BARGMANN 1998; SOKOLOWSKI 2001; LIM *et al.* 2004; KOHL *et al.* 2013). It is also well understood that differences in the development of neural architecture can result in behavioral diversification of innate behaviors as in the case of sexually dimorphic behaviors (KOHL *et al.* 2013). How these robust behaviors are fine-tuned in response to rapidly changing environmental stimuli remains vaguely answered. What are the key players that regulate the balance between maintenance of the major characteristics of innate behaviors and the acquisition of appropriate stimulus-dependent adaptations? Traditionally, proteins involved in mechanisms such as transcriptional regulation and the enzymatic components of signal transduction pathways have mostly been associated with such adaptive responses (STOCK *et al.* 1989; ABRAHAM 2008; ZOVKIC *et al.* 2013). Recently, however, miRNAs have emerged as important mediators of rapid and widespread changes in cells' transcript and proteomic content in response to environmental fluctuations (LEUNG AND SHARP 2010; NESLER *et al.* 2013). miRNAs are 19-20 nt, non-coding RNAs that target messenger RNA (mRNA) transcripts for translation inhibition, making miRNA-mediated translational control a fast mode of protein expression regulation (IFTIKHAR AND CARNEY 2016). miRNAs act by binding to

the 3'UTR of their target mRNAs and disrupting translation via the formation of RISC (RNA Induced Silencing Complex). Complex formation can be followed by any of several proposed modes of miRNA-mediated translation inhibition such as recruitment of deadenylation factors that destabilize the mRNA-miRNA duplex by removing the poly(A) tail or by blocking the circularization of mRNA that facilitates translation initiation (CARTHEW AND SONTHEIMER 2009).

Although it is known that miRNAs are expressed in the central nervous system (CNS) (MOREL *et al.* 2013; YOU *et al.* 2018) this newer potential regulatory mechanism has not been well-studied in the context of how miRNA activity can modulate behavior. Some examples of work that sheds light on roles for miRNAs in regulating behavior include studies on honeybees demonstrating that differential expression of miRNAs is linked to behavioral plasticity (GREENBERG *et al.* 2012). Work on zebra finches shows that *miR-9* and *miR-140-5p* are upregulated as a function of song behavior in the brain regions associated with vocal learning (SHI *et al.* 2013). In *D. melanogaster*, recent reports have begun to describe the role of miRNAs in behaviors such as learning, memory and circadian rhythms (BUSTO *et al.* 2015; YOU *et al.* 2018). An example is *miR-92a*, which modulates excitability of pacemaker neurons that regulate circadian rhythms (CHEN AND ROSBASH 2017). A further piece of evidence for the contribution of miRNAs to Drosophila behavior comes from the work of Picao-Osorio et al. (2015), which demonstrated that a single Drosophila miRNA (*miR-iab4*) affects larval self-righting (SR) behavior-- a movement used by the larva to correct its orientation if turned

upside-down. In their screen for SR behavior, a surprising 40% of all miRNAs tested impacted the behavior (PICAO-OSORIO *et al.* 2017).

We sought to understand the role of miRNAs in the regulation of another innate but stimulus-modulated behavior displayed by Drosophila males-- courtship behavior. Drosophila courtship is comprised of a series of robust, stereotypical steps but is constantly modified based on information received from the courtship subject (EJIMA *et al.* 2005) that includes, for example, female rejection behaviors and aversive or attractive pheromones. The potential for Drosophila to perform sex-specific reproductive behaviors is set by the production of the transcriptional regulator proteins Fruitless (FRU) and Doublesex (DSX). The sex-determination gene hierarchy is differentially initiated in females and males due to female-limited expression of *Sex-lethal (Sxl)*, resulting in sex-specific splicing of *transformer (tra)*. Expression of *tra*, in turn, determines the sex-specific expression of *fru* and *dsx* transcripts and their protein products (DAUWALDER *et al.* 2002). Expression of the male-specific form of the FRU protein (FRU^M) enables male-specific courtship and mating behaviors. Similarly, sex-specific splicing of the primary transcript for *dsx* leads to production of either a male-specific or female-specific protein isoform of Doublesex, DSX^M and DSX^F, respectively (YAMAMOTO AND KOGANEZAWA 2013). FRU proteins are proposed to transcriptionally regulate several target genes through chromatin modification, thereby turning on the genetic program required for masculinization in FRU^M-expressing neurons (ITO *et al.* 2012). Neurons that express FRU^M develop male typical structures and projections, whereas in the absence of FRU^M only female-type neurons develop (KIMURA *et al.* 2005;

YAMAMOTO AND KOGANEZAWA 2013). These sex-specific differences in the number and architecture of neurons occur in specific groups of cells such as the PC1 cluster in the posterior region of the adult brain. Several studies collectively suggest that the PC1 neuron cluster predominantly controls initiation of male courtship behavior (KIMURA *et al.* 2005; KIMURA *et al.* 2008; KOHATSU *et al.* 2011). The male-specific development of this cluster requires not only the presence of FRU^M but also the absence of DSX^F (KIMURA *et al.* 2008). Reports demonstrating that artificial activation of *fru*-expressing or *dsx*-expressing neurons induces male courtship behavior in solitary males have clarified the necessity of both *fru* and *dsx* for displaying the full complement of male courtship behaviors (PAN *et al.* 2011).

In addition to what is known about the role of proteins of the sex-determination hierarchy, recent work highlights the possible role of miRNAs in maintaining sexual identity (FAGEGALTIER *et al.* 2014). The *let-7* miRNA was shown to restrict the expression of sex-determination genes to the sex in which they are normally expressed. Fagegaltier et al. (2014) found that the levels of a downstream target of DSX^F, *Yp1,* are reduced in female flies mutant for *let-7.* In male *let-7* mutants, researchers detected spurious expression of female-specific transcripts *Sxl* and *Yp1*, indicating that *let-7* regulates sex-specific expression levels of these two transcripts as well. The role of *let-7* in sex determination is also dependent on signaling by the insect steroid hormone ecdysone. Ecdysone signaling regulates a vast array of biological responses in Drosophila including courtship behaviors (DALTON *et al.* 2009). Thus, steroid-coupled regulation of miRNAs that interact with proteins of the sex-determination hierarchy

suggests a possible role for miRNAs in regulating not only physical sexual characteristics but also reproductive behaviors.

We were therefore interested in the extent to which miRNAs influence sex-specific behaviors such as male courtship. Thus far, one study identified a role for *miR-124* in suppressing male-male courtship and enhancing male attractiveness to females. *miR-124* mutant males have elevated levels of tra^F and altered levels of male-specific pheromones (WENG *et al.* 2013). In our study we conducted a genetic screen to identify miRNAs that influence *Drosophila melanogaster* male-to-female courtship behavior. We identified numerous miRNAs that modulate behavior, and confirmed two miRNAs, *miR-263b* and *miR-317*, that affect stereotypical parameters of male-female courtship and one, *miR-957*, that suppressed male-male courtship. Our further characterization of the courtship behavior of *miR-957* mutants indicates that the males are attracted to animals with high levels of male-aversive pheromones on their bodies. Therefore, miRNAs appear to play a role in modulating sex-specific responses to pheromones.

2.2 Methods

2.2.1 Fly Stocks

A library of 80 targeted miRNA knockout (KO) strains covering 104 miRNA genes (CHEN *et al.* 2014b) was obtained from the Bloomington Drosophila Stock Center (BDSC) (http://fly.bio.indiana.edu/). The combined set of strains deletes 130 miRNA-encoding loci either singly or in clusters. *w1118* flies obtained from BDSC were used as controls (CHEN *et al.* 2014b). Strains in the *yw* background were not tested in the screen

since *yellow* mutants have been shown to have abnormal courtship behaviors (WILSON *et al.* 1976), and we found during our testing that mutants in the *yw* background had low courtship. miRNA sponge lines (*UAS-miR-957.sponge/CyO; UAS-miR-957.sponge* (BDSC#61443)*, UAS-miR263b.sponge/CyO; UAS-miR-263b.sponge* (BDSC#61403)*, UAS-miR-317.sponge/CyO; UAS-miR-317.sponge.TM6B,Tb*^{1} (BDSC#61434), *UAS-miR-31a.sponge; UAS-miR-31a.sponge* (BDSC#61383)*, UAS-scramble.sponge; UAS-scramble.sponge* (control) (BDSC#61507) (FULGA *et al.* 2015), and miRNA expression lines (*UAS-mir-957/CyO* (BDSC#60609)*, UAS-miR-317/CyO* (BDSC#59913)*, UAS-miR-310/TM3, Sb* (BDSC#41155)*, UAS-miR-310,311,312,313/TM3, Sb* (BDSC#41135)*, UAS-miR-31a/TM3, Sb, Ser* (BDSC#59869) (BEJARANO *et al.* 2012) were also obtained from BDSC. The driver lines used were *dsx-gal4/TM6B, Tb* (BDSC#66674), *fru-gal4/TM3, Sb* (gift from Barry Dickson), *elav-gal4/CyO* (BDSC#8765)*, Ubi-gal4/CyO* (BDSC#32551), and *actin-gal4/SM6b*. Chromosomal deficiency lines that removed specific miRNAs were also obtained from BDSC. *miRNA-310,311,312,313*: *Df(2R)BSC701/SM6a* (BDSC#51327), *miRNA-317*: *Df(3R)ED5454/TM6C, cu, Sb* (BDSC#9080), *miRNA-263b*: *Df(3L)BSC575/TM6C, Sb, cu* (BDSC#27587), *miRNA-31a*: *Df(2R)BSC347/CyO* (BDSC#24371), and *miRNA-957*: *Df(3L)BSC420/TM6C, Sb, cu* (BDSC#24924). *Drosophila simulans* were a gift from Mariana Mateos (Texas A&M University). *Cre D[*]/TM3, Sb* (BDSC#851) and mushroom body driver *D52H-gal4* (on the X chromosome) were provided by Paul Hardin (Texas A&M). Flies were maintained at 25°C on a 12 hr light/dark cycle on a standard cornmeal, sugar and agar diet.

2.2.2 Screen Design

We screened miRNA KO strains (in the *w1118* background) for defects in male courtship behavior. *w1118* flies were used as controls. Out of the 80 strains available, only the 60 KO strains that produced homozygotes were tested. During the course of the screen, stocks that showed very low viability of homozygotes were also dropped if a sufficiently large sample size was hard to attain (57 total strains were tested; see supplemental Table 1). Single-pair mating assays (described below) were conducted in batches of 5-10 genotypes, and a control group of *w1118* flies was tested in each batch. A sample size of at least 25 pairs was obtained for each genotype. All assays were conducted in dark conditions to remove the influence of visual cues on mating behavior. The parameters scored for all lines were courtship latency, mating latency and mating success. Courtship latency is defined as the time from the introduction of the courtship subject to the beginning of courtship. Mating latency is calculated as the elapsed time from the introduction of the female subject to the start of mating. Mating success is the proportion of pairs that mate within the 1 hr timeframe for the assay.

From the videos of the single-pair mating assays obtained during the screen, courtship index towards the female subjects was calculated for *miR-263b, miR-278* and *miR-957* KO males because their courtship appeared to be very vigorous in the assay recordings. *miR-957* also males displayed inter-male courtship when kept in groups of males. Courtship index is defined as the proportion of time spent by the male courting the courtship subject as a proportion of the total time during which the behavior is evaluated. In this study, courtship index was calculated for the first 10 min from the

introduction of the courtship subject unless otherwise noted. *w1118* males were used for control comparisons.

For strains that showed a significantly altered value for one or more of the parameters mentioned above, validation of the phenotype was performed by placing each miRNA KO allele *in trans* with a deficiency chromosome that removed the miRNA. Phenotypes observed in homozygous miRNA KO animals were evaluated in these transheterozygous animals.

2.2.3 Single-pair mating assays

A 5-day-old virgin male (miRNA mutant for the experimental condition and *w1118* for the control) was placed in a mating chamber with a 5-day-old virgin female (*Canton-S*) in dark conditions at room temperature. Behavior was recorded for 1 hr using JVC-HDD Everio cameras, and we calculated the following parameters: courtship latency, mating latency, courtship index and mating success for each pair. The small number of pairs that did not court or mate within the hour were not included in our calculations. All experiments were carried out over several days, and control and experimental animals were always tested simultaneously.

2.2.4 miRNA sponge experiments

UAS-miR.sponge lines for *miR-263b* and *miR-317* were crossed with *actin-gal4* to observe the effects of ubiquitous repression of the miRNAs. These miRNAs were specifically tested because the phenotypes observed for their KO mutants were validated

with deficiency lines. Males expressing a *UAS-scramble.sponge* sequence were used as controls for every case (FULGA *et al.* 2015). For *actin-gal4>UAS-miR-263b.sponge* males, single-pair mating assays were performed with females in the dark and the courtship index was calculated. For *actin-gal4>UAS-miR-317.sponge* males, single-pair mating assays with females were performed in the dark and mating success was calculated.

2.2.5 Rescue experiments

Elav-gal4 was used to express *UAS-mir-317* in *miR-317* KO/Df background. Single-pair courtship assays were performed to calculate mating success for the *miR-317* rescue flies and compared with *miR-317* KO/Df flies. Rescue experiments were not performed for *miR-263b* because overexpression lines were not available. We attempted to rescue expression for *miR-317* using the ubiquitous drivers *ubi-gal4* and *actin-gal4,* but expression with these drivers resulted in lethality in KO backgrounds for both miRNAs.

2.2.6 Activity measurement

For targets from the screen that were selected for further evaluation, general activity levels were compared for miRNA mutants and controls by recording the frequency at which the males crossed a line through the center of the mating chamber in the final minute before mating was achieved (MCROBERT *et al.* 2003). We did not detect activity differences for miRNA males compared to controls (Figure 3).

2.2.7 Statistics

Statistical analysis for behavior was done using JMP Pro statistical software (JMP® Pro, Version <*13.1.0*>. SAS Institute Inc., Cary, NC, 1989-2007). GraphPad Prism version 7.00 for MAC OS X, GraphPad Software, La Jolla California USA, www.graphpad.com was used for making graphs. Values for courtship latency and mating latency from the screen were log transformed, and ANOVA followed by a post-hoc Dunnett's test was performed for each batch of genotypes tested. Courtship indices were arcsine transformed and compared using a t-test to compare between two groups. A non-parametric Mann-Whitney test was used to compare the courtship index between two samples when the test for normality failed. The Chi-square statistic was calculated to compare mating success of two samples.

2.3 Results

2.3.1 miRNA KO screen

In the initial screen, we identified significant effects from removal of individual or clusters of miRNAs on several robust steps of male *Drosophila* courtship behavior (Table 1 and Table 2). Most prominently, for 16 strains we detected effects on mating success. *miR-1017* males did not mate (N=20), and males from a single KO strain, *miR-285*, had a higher mating success than the control males (N=41, P=0.0105). Out of the 15 KO strains for which males had reduced mating success, three strains (*miR-317, miR-31a* and *miR-310-311-312-313*) also displayed increased mating latency, meaning that males from these strains took longer to achieve copulation (*miR-317*: N=26, P=0.0295;

miR-31a: N=24, P=0.0327; *miR-310-311-312-313*: N =27, P=0.0143). No other strains from the 57 strains tested had differences in mating latency. However, *miR-31a* mutants also took longer to initiate courtship towards females (N=24, P=0.0368).

For courtship latency, we detected differences in both directions (increased as well as decreased courtship latency). Overall, 9 miRNA mutant strains displayed a significant increase in courtship latency, and 10 strains had reduced courtship latency (Table 1).

We noticed that *miR-263b* mutants appeared to court females extremely vigorously though we did not detect a difference in the mating latency. We then calculated the courtship index for this strain and found that it was increased (Mann-Whitney U-Test, N=27 $p<0.0001$).

	miRNA	**Courtship Latency**	**Mating Latency**	**Mating Success**
1	miR-306-79-9b	N.S. N=23	N.S. N=24	**Lower mating success, N=41, p=0.005243**
2	miR-9c	N.S. N=25	N.S. N=27	N.S. N=40

Table 1. Complete list of miRNA KO mutants tested for courtship parameters. miRNA KO mutants were tested for courtship latency, mating latency and mating success. Courtship index was also calculated for *miR-263b*, *miR-278* and *miR-957*. For strains with rare or no homozygotes the assays were discontinued. Significantly different values are in bold. N.S denotes not significant.

	miRNA	**Courtship Latency**	**Mating Latency**	**Mating Success**
3	miR-10	N.S. N=29	N.S. N=29	N.S. N=39
4	miR-11	N.S. N=29	N.S. N=27	N.S. N=40
5	miR-124	**Decreased courtship latency, N=25, p<0.0001**	N.S. N=25	N.S. N=44
6	miR-133	N.S N=29	N.S N=29	N.S N=42
7	miR-137	N.S N=29	N.S N=29	N.S N=48
8	miR-184	N.S N=30	N.S N=30	N.S N=50
9	miR-193	N.S N=27	N.S N=27	N.S N=40
	miRNA	**Courtship Latency**	**Mating Latency**	**Mating Success**
10	miR-219	N.S N=23	N.S N=23	**Lower mating success, N=48, p=0.00106**
11	miR-263b	N.S N=30	N.S N=27	N.S N=31
12	miR-274	**Decreased courtship latency, N=24, p<0.0001**	N.S N=30	N.S N=41
13	miR-276b	Less than 20 mated flies	Less than 20 mated flies	**Lower mating success, N=36, p=0.000001**
14	miR-278	**Increased, N=32, p=0.014**	N.S N=36	N.S N=59
15	miR-282	N.S N=25	N.S N=27	**Lower mating success, N=43, p=0.027509**
16	miR-283	N.S N=29	N.S N=31	N.S N=42
17	miR-285	N.S N=29	N.S N=37	**Higher mating success, N=41 p=0.0105**

Table 1. continued

	miRNA	Courtship Latency	Mating Latency	Mating Success
18	miR-2a-2-2a-1-2b-2	**Decreased courtship latency, N=26, p<0.0001**	N.S N=29	**Lower mating success, N=50, p=0.026092**
19	miR-2b-1	N.S N=34	N.S N=34	N.S N=46
20	miR-2c, miR-13a, miR-13b-1	N.S N=29	N.S N=24	N.S N=38
21	miR-303	**Increased courtship latency, N=30**	N.S N=30	N.S N=42
22	miR-304	N.S N=29	N.S N=29	N.S N=37
23	miR-307a-307b	**Decreased courtship latency, N=27, p=0.0222**	N.S N=28	**Lower mating success, N=45, p=0.012463**
24	miR-310-311-312-313	N.S N=27	**Increased mating latency, N=27, p=0.0143**	**Lower mating success, N=70, p=0.000001**
25	miR-316	**Decreased courtship latency, N=23, p<0.0001**	N.S N=23	**Lower mating success, N=64, p=0.000001**
26	miR-317	N.S N=26	**Increased mating latency, N=26, p=0.0295**	**Lower mating success, N=50, p=0.01508**
27	miR-318	**Decreased courtship latency, N=26, p<0.0001**	N.S N=26	**Lower mating success, N=51, p=0.014699**

Table 1. continued

	miRNA	Courtship Latency	Mating Latency	Mating Success
28	miR-31a	**Increased courtship latency, N=24, p=0.0368**	**Increased mating latency, N=24, p=0.0327**	**Lower mating success, N=66, p=0.000001**
29	miR-31b	N.S N=32	N.S N=32	N.S N=45
30	miR-33	N.S N=26	N.S N=26	N.S N=38
31	miR-87	N.S N=24	N.S N=24	**Lower mating success, N=43, p=0.28444**
32	miR-929	Less than 20 mated flies	Less than 20 mated flies	**Lower mating success, N=53, p=0.00**
33	miR-92a	N.S N=29	N.S N=29	N.S N=37
34	miR-92b	**Increased courtship latency, N=27, p=0.0166**	N.S N=27	**Lower mating success, N=61, p=0.004183**
35	miR-932	**Decreased courtship latency, N=22, p=0.0062**	N.S N=23	N.S N=42
36	miR-955	**Decreased courtship latency, N=26, p=0.0381**	N.S N=26	N.S N=38
37	miR-956	**Decreased courtship latency, N=25, p<0.0001**	N.S N=25	**Lower mating success, N=47, p=0.010612**
38	miR-957	N.S N=29	N.S N=29	N.S N=38
39	miR-958	N.S N=29	N.S N=29	N.S N=38
40	miR-959-960-961-962	N.S N=27	N.S N=27	N.S N=48

Table 1. continued

	miRNA	Courtship Latency	Mating Latency	Mating Success
41	miR-965	N.S N=28	N.S N=28	N.S N=35
42	miR-966	N.S N=30	N.S N=30	N.S N=42
43	miR-967	**Decreased courtship latency, N=26, p<0.0001**	N.S N=26	N.S N=33
44	miR-970	N.S N=27	N.S N=27	N.S N=34
45	miR-971	**Increased courtship latency, N=28**	N.S N=28	N.S N=40
46	miR-975	N.S N=29	N.S N=29	N.S N=36
47	miR-980	N.S N=26	N.S N=26	N.S N=29
48	miR-984	**Increased courtship latency, N=30**	N.S N=30	N.S N=44
49	miR-986	**Increased courtship latency, N=31, p=0.0290**	N.S N=31	N.S N=54
50	miR-987	N.S N=26	N.S N=26	N.S N=37
51	miR-988	**Increased courtship latency, N=25, p=0.0003**	N.S N=28	N.S N=46
52	miR-989	N.S N=24	N.S N=29	N.S N=51
53	miR-990	N.S N=25	N.S N=25	N.S N=39
54	miR-999	N.S N=26	N.S N=26	N.S N=39
55	miR-1007	**Increased, N=29, p=0.0003**	N.S N=29	N.S N=33
56	miR-1014	N.S N=29	N.S N=32	N.S N=36
57	miR-1017	**-**	**-**	**No mating, N=20**

Table 1. continued

2.3.2 Validation of candidates

We selected candidates from the initial screen to determine if the observed phenotypes were maintained when we placed the KO allele *in trans* with a chromosomal deficiency (Df) for the miRNA (Table 2). From the strains with reduced mating success, we selected those that also had increased mating latency and/or increased courtship latency for validation with deficiencies. Namely, these were *miR-317, miR-31a and miR-310-311-312-313*. Transheterozygotes for *miR-317*, like the KO mutants, had lower mating success (Chi-Square, N=52, P=0.004973). However, they did not have a significant increase in mating latency. Hetero-allelic mutants for *miR-31a* and *miR-310-311-312-313* did not affect mating success or mating latency.

miR-263b KO mutant males had a higher courtship index than controls, as did *miR-263b* transheterozygous mutants (Mann-Whitney U-Test N=29, P<0.00001). *miR-285* and *miR-1017* KO/Df males did not have the originally observed phenotypes.

Finally, we further validated the phenotypes observed in *miR-317* and *miR-263b* KO mutants by expressing miRNA sponges to reduce the expression of *miR-317* and *miR-263b* under the control of the ubiquitous *actin-gal4* driver. For *actin-gal4>UAS-miR-317.sponge* males, mating success was significantly lower than for the *actin-gal4>UAS-scramble.sponge* controls (N=36, P=0.0406). For *actin-gal4>UAS-miR-263b.sponge* males, courtship index was higher than for the *actin-gal4>UAS-scramble.sponge* controls although the difference was not significant (N=28, P=0.0594).

Expression of *miR-317* was restored via the pan-neuronal driver *elav-gal4* in the *miR-317* KO background, and the reduced mating success phenotype was not rescued

(N=40, P=0.1791). We were unable to test for rescue by ubiquitous expression of the miRNA due to lethality.

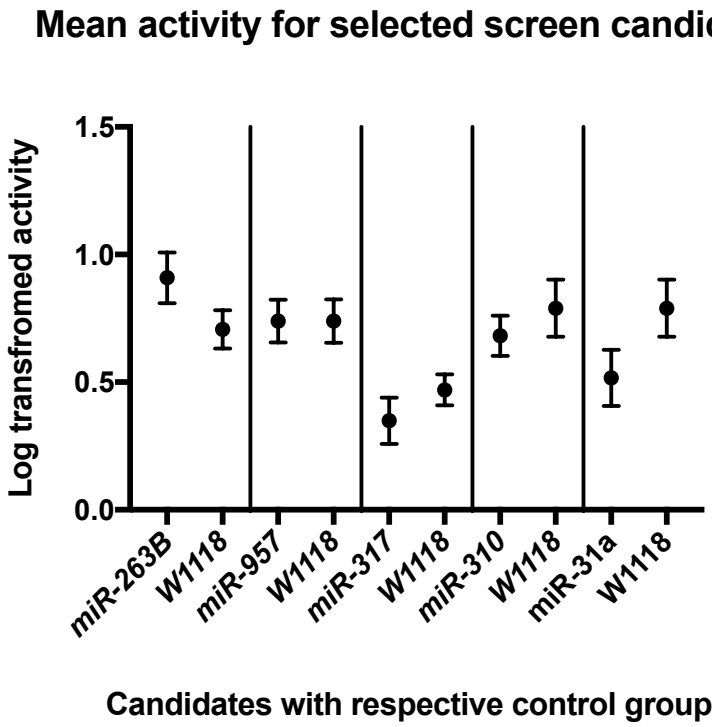

Figure 3. Activity measurements of miRNA KO mutant screen candidates. Activity levels of experimental animals and controls from their respective batches were evaluated by counting the number of times flies crossed a line placed across a chamber in the minute before mating. Error bars represent mean ± standard error (SEM).

miRNA	Phenotype observed in KO	Phenotype with transheterozygote (KO/Df)	Phenotype observed in both mutants
miR-263b	Increased courtship index (N=27, P<0.0001)	Increased courtship index (N=29, P<0.00001)	✓
miR-957	Male-male courtship and chaining (N=10 x3, P=0.0024)	Male-male courtship and chaining (N=10 x3, 0.0189)	✓
miR-317	Increased mating latency (N=26, P=0.0295) Lower mating success (N=50, P=0.01508)	Mating latency not affected (N=30, P=0.6615) Lower mating success (N=52, P=0.004973)	✗ ✓
miR-310,311,312,313	Increased mating latency (N=27, P=0.0143) Lower mating success (N=70, P=0.000001)	Mating latency not affected (N=29, P=0.6362) Mating success not affected (N=35, P=0.8286)	✗
miR-31a	Increased courtship latency (N=24,P=0.0368) Increased mating latency (N=24, P=0.0327) Lower mating success (N=66, P=0.000001)	Courtship latency not affected (N=30, P=0.2472) Mating latency not affected (N=30, P=0.3103) Mating success not affected (N=30, P=0.126)	✗
miR-285	Higher mating success (N=41, P=0.0105)	Mating success not affected (N=49, P=0.5632)	✗
miR-1017	No mating (N=20)	Mating success not affected (N=20, P=0.3865)	✗

Table 2. miRNA KO mutants tested for courtship parameters. miRNA KO mutants with significant variations in courtship and/or mating parameters were further tested as KO/Df transheterozygotes. *miR-957* KO mutants were backcrossed into the *Canton-S* background after removal of the *mini-white* gene and then tested for male-male courtship and chaining behavior.

2.4 Discussion

2.4.1 miRNA KO screens in *Drosophila melanogaster* are a powerful genetic tool for understanding behavior

miRNAs have been implicated in subtle regulation of protein expression, for example, by acting as a mechanism to counter the effects of leaky gene expression (LAI *et al.* 2016). Yet, several recent studies have begun to emphasize the role of miRNAs in the regulation of more complex behavioral phenotypes such as learning (WANG *et al.* 2012b; LI *et al.* 2013; BUSTO *et al.* 2015), rhythmic behavior (YOU *et al.* 2018), egg-laying

(FRICKE *et al.* 2014; ZHOU *et al.* 2014; NEHAMMER *et al.* 2015) and locomotor activity (FULGA *et al.* 2015; NEHAMMER *et al.* 2015; PICAO-OSORIO *et al.* 2017). The impact of miRNAs on these behaviors result from the cumulative effect of altered regulation of several mRNA targets. Most studies have explored the effects of miRNA null mutations (LI *et al.* 2013; CHEN *et al.* 2014b; PICAO-OSORIO *et al.* 2017), and in several cases these effects were observed not just during early stages of development but also in adult organisms (LI *et al.* 2013; WENG *et al.* 2013; CHEN *et al.* 2014b). These results show that miRNAs may impact levels of several proteins within the cell without being essential for the organism's viability. Hence, miRNA KO screens provide a powerful genetic tool to observe phenotypes resulting from complete genetic ablation of a post-transcriptional regulatory element while retaining viability of the subjects. We utilized this tool to understand the extent to which miRNAs contribute to a complex behavior in *Drosophila melanogaster* by assessing effects of single KO alleles on aspects of male courtship and reproductive behavior.

Understanding the factors that dictate social behavior such as male courtship involves both analyzing the external stimuli and the body's response mechanisms. To understand the genetic basis of these responses, studies have previously shown that social interactions can induce rapid changes in the transcriptomic profiles of *Drosophila melanogaster* males (ELLIS AND CARNEY 2009). Moreover, evidence suggests that these gene expression patterns are influenced by the sex of the interacting partner (ELLIS AND CARNEY 2011), thereby showing that stimulus-response dynamics are more complex than previously assumed. Drosophila courtship behavior is an innate behavior primarily

controlled by neurons that express major transcription factors of the fly sex-determination hierarchy. This circuit is responsive to sensory stimuli, and courtship behaviors can be modified based on the attractive or aversive nature of the stimuli received. One of the most potent contributors to this regulation is the complex complement of stimulatory or inhibitory pheromones (KOHL *et al.* 2015; SCHULTZHAUS *et al.* 2017). In our current study we identified miRNAs that impacted one or more of the stereotypical parameters of courtship. In the initial screen ~26% of the strains tested had reduced mating success, while ~39% showed variations in other parameters. *miR-957*, in particular, was shown to be important for the detection of male-aversive pheromones. The absence of the miRNA led to compromised sensing and evaluation of the courtship targets. To our knowledge, there have been no published genome-wide screens to assess the function of individual miRNAs in regulating courtship behavior.

2.4.2 *miR-263B* and *miR-317* impact important parameters of male-female courtship

Our screen identified additional miRNAs that affect various aspects of male courtship and mating behaviors. *miR-263B* is a conserved miRNA (mammalian orthologs are in the *miR-183* family) that was recently characterized in detail as part of a study to identify astroglial miRNAs that regulate circadian behavior (YOU *et al.* 2018). *miR-263b* is widely expressed in the CNS and has experimentally validated targets including *hid* and the in-silico predicted target *Clock* (YOU *et al.* 2018). In our study *miR-263b* mutants showed increased courtship directed towards wild-type females, and this phenotype was

also observed with *miR-263b* transheterozygous mutants and sponges. The increase in courtship activity of *miR-263b* suggests that it is involved in the suppression of proteins that enhance courtship activity. No experimentally validated targets exist, and *fru* is not a predicted target for *miR-263b* in TargetScan, a program that calculates potential mRNA targets of miRNAs (RUBY *et al.* 2007).

miR-317 males showed reduced mating success and the phenotype was observed in the KO mutants as well as the KO/Df transheterozygotes. Males expressing the *miR-317*-sponge also showed the same phenotype. However, neuronal expression of *miR-317* expression with *elav-gal4* was not sufficient to rescue the mutant phenotype. Previously, *miR-317* has been implicated in locomotor behavior, startle response, morphogenesis (YAMAMOTO *et al.* 2008) and inter-male aggressive behavior (EDWARDS *et al.* 2009). Additionally, *miR-317* has been associated with effects on female post-mating responses (FRICKE *et al.* 2014).

2.4.3 Emerging roles for miRNAs in the regulation of complex behaviors

Several recent studies have documented the role of miRNAs in regulation of transcript levels for maintenance of complex phenotypes (VARGHESE *et al.* 2010; VODALA *et al.* 2012; WENG *et al.* 2013; BUSTO *et al.* 2015; PICAO-OSORIO *et al.* 2017; YOU *et al.* 2018). A complex and robust behavior that has not received as much attention in the context of miRNA mediated regulation is courtship behavior. By conducting a genetic screen with miRNA KO mutants, we showed that individual miRNAs can influence one or more aspects of male courtship behavior. miRNAs may be contributing to courtship

behavior through involvement in the maintenance of site and sex-specific protein expression by repressing the translation of transcripts produced from leaky expression of genes at sites where the expression of these genes needs to be limited. For example, Weng et al. (2013) demonstrated that a modest increase in the level of the female-specific *tra* splice form, tra^F, in *Drosophila melanogaster miR-124* mutant males led to reduced splicing of *dsx* which led to decreased production of the male-specific isoform of *dsx* (*dsxM).*

miRNAs are recognized as being important for adaptive responses to environmental changes (HE *et al.* 2016). Therefore, apart from restricting leaky expression, miRNAs are ideal candidates for factors that bring about fast changes in the proteomic landscape in response to rapidly changing cues from courtship subjects. Alternatively, the effects of miRNAs on courtship behaviors could be a result of their effects of on the development of the male courtship circuitry since miRNAs have been shown to have roles in neurodevelopment and dendritic remodeling (NESLER *et al.* 2013).

Further assessment of the effects of miRNA candidates that come from screens such as ours may involve using other experimental techniques to validate targets. Recently You et al. (2018) attempted to validate targets of *miR-263b* in the context of circadian regulation by using RNAi against selective, computationally predicted targets of *miR-263b* (YOU *et al.* 2018). Their aim was to see if reduction of predicted target mRNAs of *miR-263b* produced the same phenotype (arrhythmic behavior) seen in the mutants for the behavior. Their rationale for using RNAi against targets stemmed from

the results of *miR-263b* overexpression experiments that showed that flies with *miR-263b* overexpression showed arrhythmic behavior similar to the KO mutants. Their experiments, however, did not identify targets for *miR-263b* (YOU *et al.* 2018). Other approaches that may be employed for target validation include overexpressing predicted targets and testing for phenotypes similar to those observed in the miRNA mutants.

The availability of a broad range of genetic tools for miRNA modulation in a model organism such as *D. melanogaster* (IFTIKHAR *et al.* 2017) has started a wave of research that explores the role of miRNAs in intricate behaviors needed for the organism's survival. Such studies will help in adding missing pieces to several neural regulatory pathways and elucidating the relevance of an additional layer of RNA-mediated gene expression control.

3. *miR-957* MUTANT MALES DISPLAY MALE-MALE COURTSHIP

3.1 Introduction

Previous studies explored roles of proteins in development and maintenance of sex-specific characteristics in Drosophila, but less is known about additional layers of regulation in this context, such as those imposed by non-coding RNAs. Chapter 2 presented results of a genetic screen carried out to understand how courtship parameters are affected by absence of miRNAs. We found that ~39% of tested miRNAs affect male courtship behavior. Of note, *miR-957* mutant males displayed high levels of male-male courtship. The following sections of this chapter present results that show that expression of *miR-957* in *doublesex*-expressing and mushroom body neurons is important for appropriate mate recognition. *miR-957* KO males actively courted other males rather than eliciting courtship from male flies but did not respond differently to female attractive cues. This chapter discusses evidence that suggests that reduced ability to process inhibitory pheromones from courtship subjects, particularly a gustatory pheromone, 7-T, likely accounts for male-male courtship. This study provides new insights into possible roles of miRNAs in regulation of complex physiological and behavioral processes in adult *D. melanogaster*.

3.2 Methods

3.2.1 Single-pair mating assays

A 5-day-old virgin male (miRNA mutant for the experimental condition and *w1118* for the control) was placed in a mating chamber with a 5-day-old virgin female (*Canton-S*) in dark conditions at room temperature. Behavior was recorded for 1 hr using JVC-HDD Everio cameras, and we calculated the following parameters: courtship latency, mating latency, courtship index and mating success for each pair. The small number of pairs that did not court or mate within the hour were not included in our calculations. All experiments were carried out over several days, and control and experimental animals were always tested simultaneously.

3.2.2 Removal of mini-white from *miR-957* KO strain

w[*]; TI{w[+mW.hs]=TI}mir-957[KO] males were crossed to *y[1] w[67c23] P{y[+mDint2]=Crey}1b; D[*]/TM3, Sb[1]* females. White-eyed progeny were collected and crossed twice with *w1118* flies to confirm the removal of the *mini-white* gene. The flies were then backcrossed 6 times into the *Canton-S* (*TM3, Sb/TM6B, Tb*) background and tested for male-male courtship. *TM3, Sb/TM6B, Tb* flies were used as control animals for subsequent assays with the backcrossed *miR-957* KO males.

3.2.3 Evaluating male courtship behavior of *miR-957* grouped males

Virgin *miR-957* KO males were collected within 4 hrs of eclosion and aged for 10 days. On the 10^{th} day males were aspirated into food vials in groups of 10 males 2 hrs before

"lights on". The next day the males were videoed for 10 min at "lights on" (VILLELLA *et al.* 1997). Within the group, *miR-957* KO males were observed courting in pairs (several pairs courting simultaneously) as well as in groups of 3 or more males (known as "chaining"). Since males rapidly switched from courting in pairs to courting in chains, we termed the overall behavior 'total courtship and chaining index' (tCCI). tCCI was calculated as the proportion of the assay time males spent courting in either chains or in multiple pairs. The same protocol was followed for observing courtship among males that were transheterozygous for the *miR-957* KO mutation over a corresponding deficiency.

3.2.4 Single-pair male-male courtship assays

A 5-day-old virgin male (*miR-957* KO or control) was placed in a mating chamber with a 5-day-old, virgin, decapitated *Canton-S* male in dark conditions at room temperature. Behavior was recorded for 20 min using JVC-HDD Everio cameras. We compared the courtship of *miR-957* or control males towards the decapitated males.

3.2.5 Single-pair elicitation assays

A 5-day-old virgin *Canton-S* male was placed in a mating chamber with a 5-day-old, virgin, decapitated *miR-957* male in dark conditions at room temperature. Behavior was recorded for 20 min. For control experiments, a decapitated *Canton-S* male was placed in the chamber as the courtship object.

3.2.6 Two-choice assays

A 5-day-old virgin male (courter) was placed in a mating chamber with two 5-day-old, virgin, decapitated subjects: a *Canton-S* male and similarly treated *Canton-S female*. The percentage of time the courter spent courting each subject in the first 10 min of the assay was recorded. The subject that was courted for the greater percentage of time was scored as the preferred subject.

3.2.7 Single-pair courtship assays with *D. simulans* females or *D. melanogaster* mated females

3.2.7.1 Courtship index towards *D. simulans* females or *D. melanogaster* mated females

A 5-day-old virgin male (*miR-957* or control) was placed in a mating chamber with either a 5-day-old, virgin, decapitated *D. simulans* female or a *D. melanogaster (Canton-S)* female in dark conditions at room temperature. In a second experiment, males were presented with a 5-day-old virgin or mated *D. melanogaster* female. Behavior was recorded for 30 min. Courtship index was calculated for the final 10 min of the assay.

3.2.7.2 Assessment of proportion of flies displaying courtship behavior

Pairs of flies in the above described assay were also evaluated for presence or absence of courtship (chasing, wing extensions and attempted copulation) for each minute of the last 5 min of the 30 min recording. This information was used to calculate the proportion

of males that continued to court *D. simulans* females or mated *D. melanogaster* females after more than 20 min of exposure to the female. Prior work from our lab demonstrated that *Canton-S* males significantly decrease courtship toward virgin *D. simulans* females, but not toward virgin *D. melanogaster* females, within 20 min of exposure (ELLIS AND CARNEY 2009). Males that did not court (4 males paired with *D. simulans* females) or mated within the timeframe of the assay (6 pairs spread across all pairings) were removed from the analysis.

3.2.8 Sponge experiments

The *UAS-miR.sponge* line for *miR-957* was crossed with *actin-gal4* to observe the effects of ubiquitous repression of the miRNAs. These miRNAs were specifically tested because the phenotypes observed for their KO mutants were validated with deficiency lines. Males expressing a *UAS-scramble.sponge* sequence were used as controls for every case (FULGA *et al.* 2015). For *actin-gal4>UAS-miR-957.sponge* males, tCCI was calculated for grouped males. In each case the results were compared to *actin-gal4>UAS-scramble.sponge* males.

3.2.9 Rescue experiments

For *miR-957*, expression was restored using *elav-gal4* to drive the expression of *UAS-mir-957* in neurons in the *miR-957* KO background. tCCI was calculated for the rescue animals and compared with *miR-957* KO flies. We attempted to rescue expression for

miR-957 using the ubiquitous drivers *ubi-gal4* and *actin-gal4,* but expression with these drivers resulted in lethality in KO backgrounds for both miRNAs.

3.2.10 Perfuming experiments

5-day-old virgin, *Canton-S* females were lightly anesthetized and coated with 1ul (per fly) of the pheromone solution to be tested. 7-T was diluted in hexane at a concentration of 3ug/ul and cVA was diluted at a concentration of 3ug/ul in ethanol. 7-T (≥95% pure) and cVA (>98% pure) were obtained from Cayman Chemical. Females to be used as controls were coated with 1ul of the respective solvents for each pheromone. The flies were allowed 15-20 min on the food vial to groom and recover from anesthesia and were then decapitated. The perfumed and decapitated female subjects were then placed in mating chambers with either *Canton-S* or *miR-957* 5-day-old virgin males for 30 min. Courtship index for each pair was calculated for the final 10 min of the assay.

3.2.11 Statistics

Statistical analysis for behavior was done using JMP Pro statistical software (JMP® Pro, Version <*13.1.0*>. SAS Institute Inc., Cary, NC, 1989-2007). GraphPad Prism version 7.00 for MAC OS X, GraphPad Software, La Jolla California USA, www.graphpad.com was used for making graphs. Values for courtship latency and mating latency from the screen were log transformed, and ANOVA followed by a post-hoc Dunnett's test was performed for each batch of genotypes tested. Courtship indices were arcsine transformed and compared using a t-test to compare between two groups. A non-

parametric Mann-Whitney test was used to compare the courtship index between two samples when the test for normality failed. The Chi-square statistic was calculated to compare mating success of two samples. For perfuming experiments a two-way ANOVA was applied to test for interaction between male genotype and type of female. A one-way ANOVA followed by a post-hoc Tukey Kramer's test was used for multiple comparisons (BILLETER AND LEVINE 2015).

3.3 Results

3.3.1 Characterization of *miR-957* grouped male behavior

As a part of the effort to identify aberrations in the courtship behavior of the strains being tested in our screen, in addition to performing male-female single-pair courtship assays, we also looked for signs of male-male courtship among grouped males of each strain. Most strikingly, we noticed that groups of *miR-957* KO males displayed high levels of male-male courtship. Since the original *miR-957* KO allele contains a copy of the *mini-white* gene, which is known to increase male-male courtship interactions in some contexts (KRSTIC *et al.* 2013), we removed the *mini-white* locus and evaluated the resulting *miR-957* KO males for male-male courtship. After removal of *mini-white*, *miR-957* KO males continued to court one another vigorously while control males performed little male-male courtship (3 groups of 10 males for each genotype; N=30 per genotype; Mann-Whitney U-Test, P= 0.0024, Figure 4)

When 10-day-old males were stored in food vials in groups of 10 males, they courted vigorously. Two patterns of behaviors were observed. Firstly, several pairs of

males were seen courting simultaneously. Pairwise courtship was frequently intermixed with chaining behavior (groups of 3 or more males courting each other). Since the males frequently shifted from courting in pairs to courting in chains, we scored the behavior as a composite of pairwise courtship and chaining behavior and termed it 'total courtship and chaining index' (tCCI). *miR-957* KO males showed significantly higher amounts of courtship compared to their wild-type counterparts (Figure 4). *miR-957* transheterozygous males continued to court males vigorously, while control males displayed little male-male courtship (3 groups of 10 males each, N=30 for each genotype; P= 0.0189). Pan-neural expression of *miR-957* via *elav-gal4* in KO males significantly reduced male-male courtship behavior (Figure 4) suggesting a role for *miR-957* neural expression in inhibiting male-male courtship interactions.

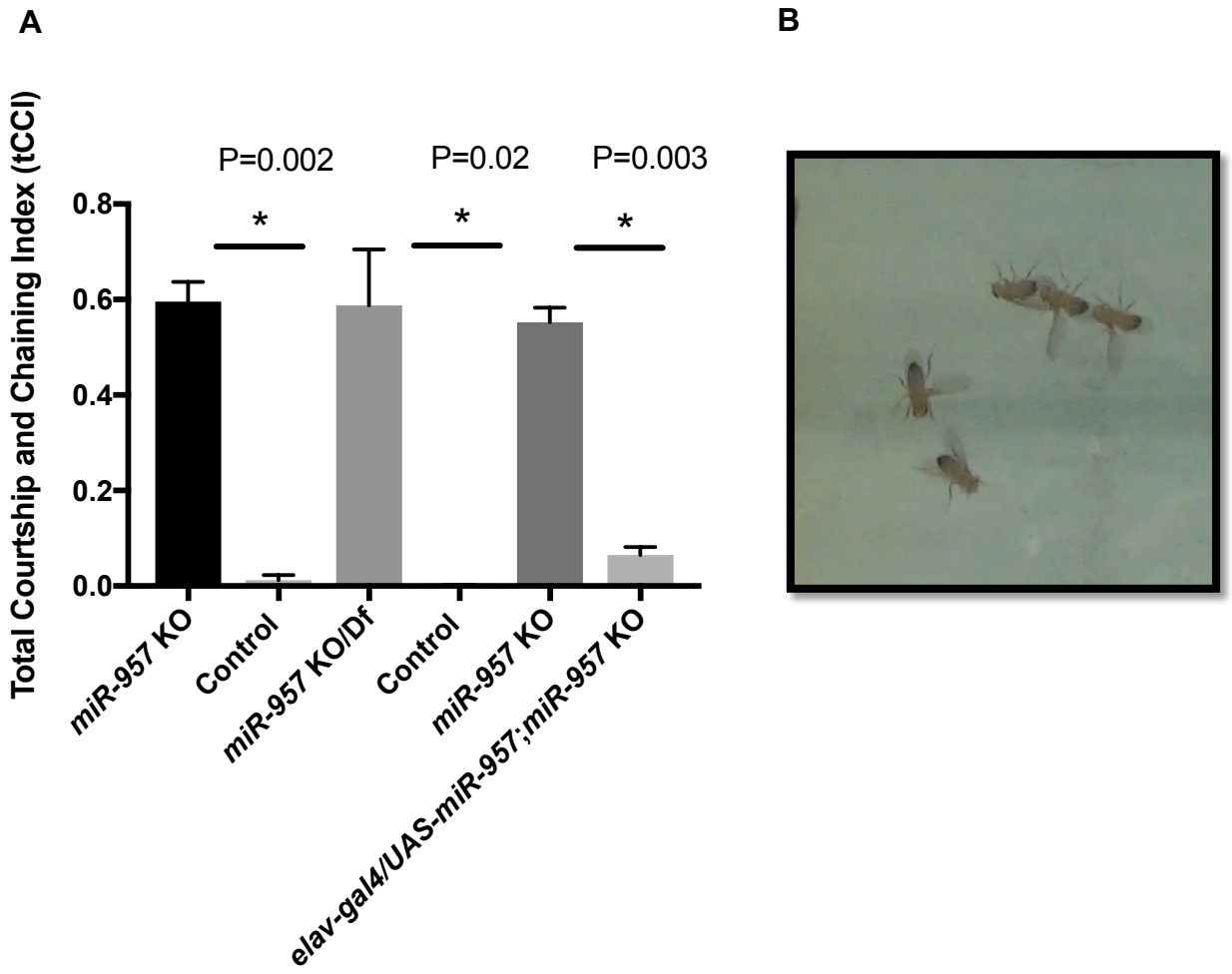

Figure 4. Total Courtship and Chaining Index (tCCI) in grouped *miR-957* KO males. A) tCCI for *miR-957* KO mutants, *miR-957* KO/Df transheterozygotes, and flies with neuronal rescue of *miR-957* compared with respective controls. Plotted tCCI values are the average of tCCI values for 3 groups of 10 males each for every genotype. tCCI values were compared between mutants and controls using t-tests. **B)** *miR-957* KO males displaying pairwise courtship or chaining. Error bars represent mean ± standard error (SEM).

3.3.2 *miR-957* reduction in *dsx* and mushroom body neurons increases male-male courtship

To further validate the male-male courtship phenotype as well as gain insight into the location of *miR-957* function, we used miRNA sponges to tissue specifically reduce *miR-957* (Figure 5). We first tested the *miR-957* sponge (FULGA *et al.* 2015) by ubiquitously expressing it using an *actin-gal4* driver. Males with ubiquitous reduction of *miR-957* performed significantly more courtship towards males than *actin-gal4>UAS-scramble.sponge* control males. We then checked if neuronal repression of *miR-957* with the pan-neuronal driver *elav-gal4* had a similar effect. The *elav-gal4>UAS-miR-957.sponge* flies actively courted males, whereas very low courtship was observed for *scramble* controls (Figure 5).

To further explore subpopulations of neurons requiring *miR-957* activity to inhibit male-male interactions, we used drivers that allowed us to express the sponge in neurons expressing two well-known regulators of courtship behavior in Drosophila-- *dsx* and *fru*. *dsx* and *fru* produce transcription factors crucial for cell sexual identity as well as sex-specific behaviors and secondary sexual characteristics (RIDEOUT *et al.* 2010; PAVLOU AND GOODWIN 2013). To our surprise, while we observed very high levels of male-male courtship in groups of *dsx-gal4>UAS-miR-957.sponge* flies, we detected little male-male courtship among *fru-gal4>UAS-miR-957.sponge* males. Since information from receptor neurons for sexual cues is ultimately relayed to higher processing centers such as the mushroom body via synapses formed in the glomeruli of the antennal lobe (ZIEGLER *et al.* 2013; SCHULTZHAUS *et al.* 2017), we also tested for effects of reducing

miR-957 in the mushroom body using *D52H-gal4* (YI *et al.* 2013). Indeed, reducing *miR-957* expression in the mushroom body also resulted in high levels of male-male courtship behavior (Figure 5).

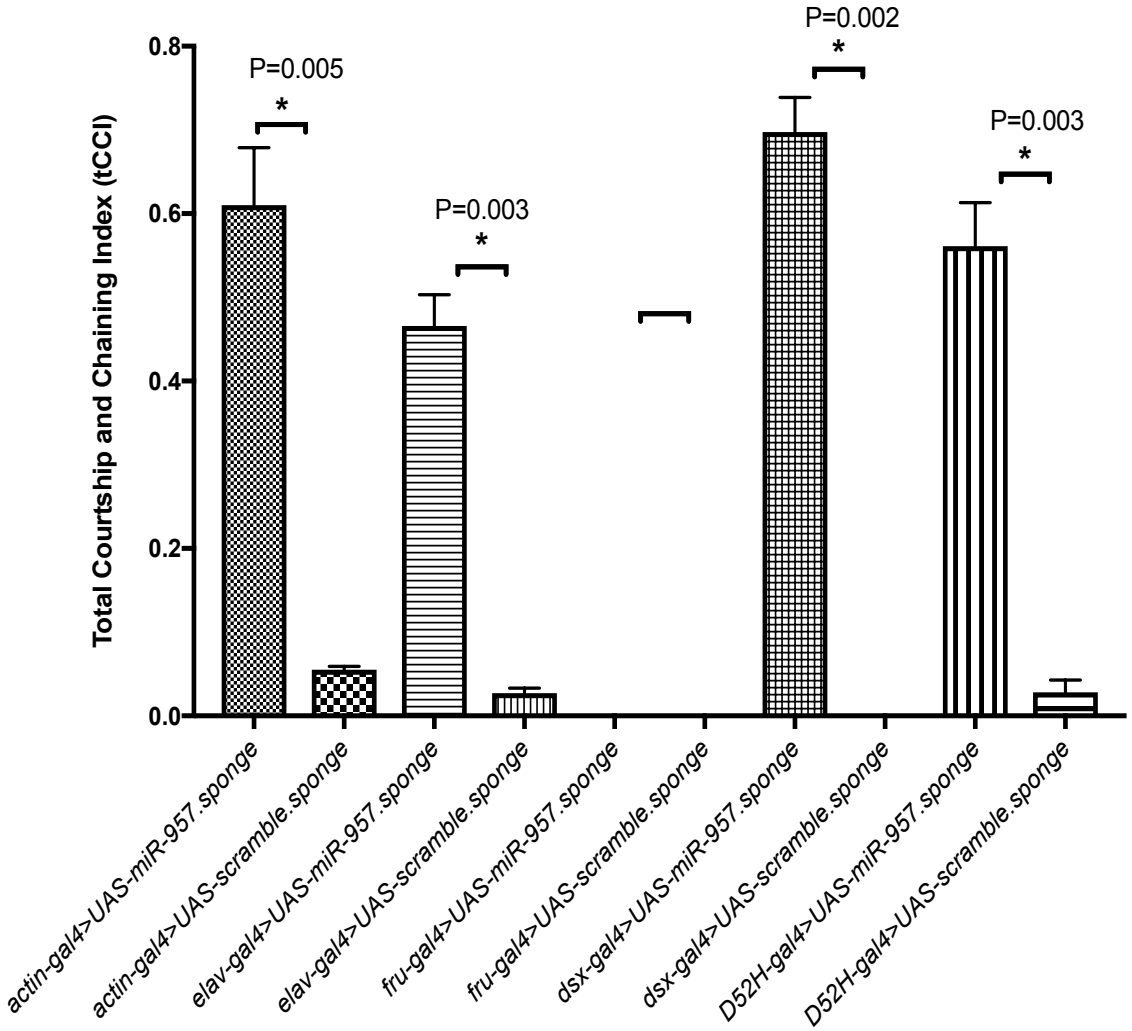

Figure 5. tCCI for grouped males with tissue-specific reduction of *miR-957.*

Ubiquitous (*actin-gal4*), pan-neuronal (*elav-gal4*), or neuron sub-population (via *fru-gal4*, *dsx-gal4*, or *D52H-gal4*) expression of *UAS-miR-957.sponge.* Plotted tCCI values are the average of tCCI values for 3 groups of 10 males each for every genotype. tCCI values for each *miR-957*.sponge and *gal4* combination were compared to the respective *scramble* control using t-tests. Error bars represent mean ± standard error (SEM).

3.3.3 *miR-957* KO males court other males but do not elicit courtship

We next wanted to understand why *miR-957* KO males court one another. The courtship could be a consequence of changes in the pheromonal profile of *miR-957* KO males that made them more attractive to other males, hence eliciting courtship. Another explanation could be a neurophysiological change that caused *miR-957* KO males to direct courtship efforts towards male subjects. To distinguish between the two possibilities, we performed courtship and elicitation assays separately (Figure 6). The courtship of *miR-957* KO males towards decapitated *Canton-S* subjects was significantly higher than that of control males (Mann-Whitney U-Test, N_1=30, N_2=25, P=0.005; Figure 3A). However, there was no significant difference between the courtship elicited by control or *miR-957* KO males from *Canton-S* males (Figure 6B). These results indicate that the courtship observed among *miR-957* grouped males was more likely a consequence of changes in their neural physiology than a change in their pheromonal profile.

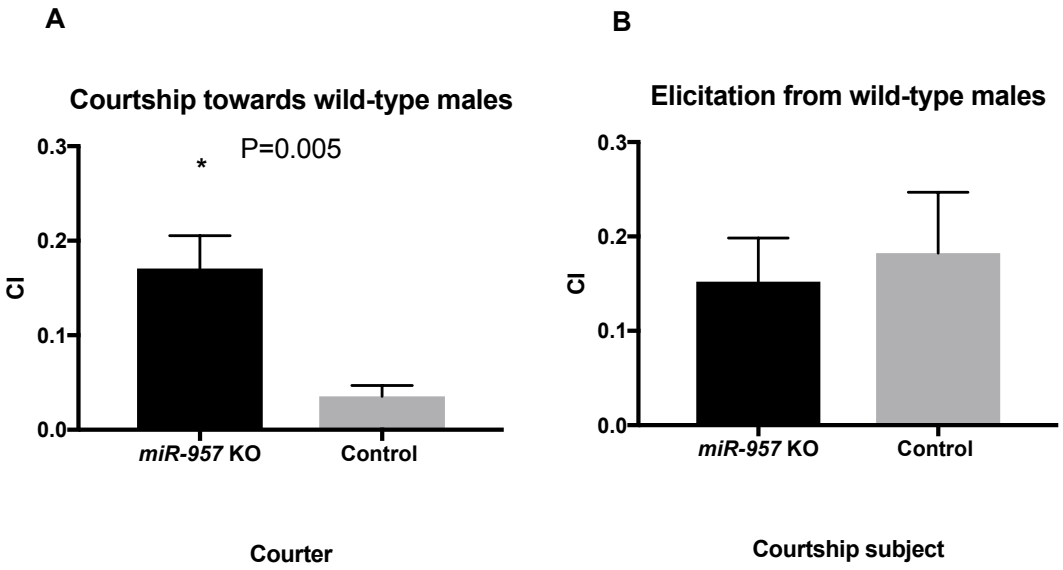

Figure 6. *miR-957* courtship and elicitation assays. A) Courtship of *miR-957* or control males towards decapitated *Canton-S* male subjects. **B)** Courtship elicited by control or *miR-957* KO males from *Canton-S* males. Courtship Index (CI) values for experimental and control groups were compared using the Mann-Whitney U-Test. N=25-30 for each genotype. Error bars represent mean ± standard error (SEM).

3.3.4 *miR-957* KO males prefer female over male subjects

We predicted that *miR-957* KO males court other males due to their inability to correctly perceive pheromones, which suggested 3 possibilities: 1) *miR-957* KO males have lost the ability to correctly perceive pheromones in general, causing them to lose their ability to distinguish between males and females, 2) *miR-957* KO males have altered perception

of male inhibitory pheromones, or 3) they are attracted more strongly to a male stimulatory cue.

We performed competition assays to help assess the first possibility. If males generally do not perceive chemical cues correctly, their ability to distinguish males from females should be impacted. When *miR-957* KO males were presented with a decapitated wild-type male and a decapitated female simultaneously under dark conditions, control and *miR-957* KO males both preferred females, indicating that *miR-957* KO males have not lost the ability to differentiate females from males (Figure 7).

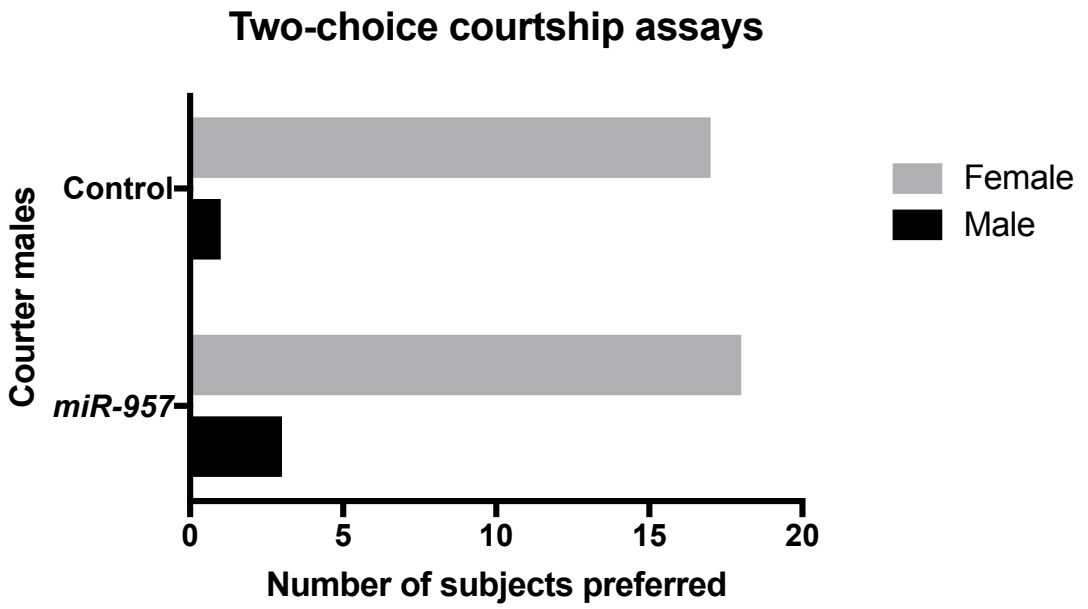

Figure 7. *miR-957* KO males prefer females. The proportion of *miR-957* and control males that choose to court females or males for the majority of a 10-min two-choice assay. Results were compared using Chi-square test. N=18 (control) and N=21 (*miR-957*).

3.3.5 *miR-957* KO males are attracted to animals with high levels of male-aversive pheromones

The results of the competition assays indicated that *miR-957* KO males respond appropriately to some chemical cues since they still prefer female courtship subjects. However, *miR-957* KO males may not detect male inhibitory cues correctly. We next tested the response of *miR-957* KO males towards two types of female subjects (mated

D. melanogaster females or *Drosophila simulans* females) known to have higher amounts of pheromones that are aversive to *D. melanogaster* males. Mated females have increased levels of male courtship inhibitory pheromones due to their mechanical transfer during copulation (SCOTT 1986; EJIMA *et al.* 2007). These include the well-characterized *cis*-vaccenyl acetate (cVA) and 7-tricosene (7-T) (KURTOVIC *et al.* 2007; LACAILLE *et al.* 2007; BILLETER *et al.* 2009; BILLETER AND LEVINE 2015; LATURNEY AND BILLETER 2016). *D. simulans* females have much higher concentrations of 7-T on their cuticles compared to *D. melanogaster* females (JALLON AND DAVID 1987), and several studies have shown that 7-T recognition is essential for preventing courtship by *D. melanogaster* males towards heterospecific females (SCOTT 1986; LACAILLE *et al.* 2007; FAN *et al.* 2013).

A larger proportion of *miR-957* KO males courted mated females, although their response to virgin females was similar to that of control males (Figure 8A). *miR-957* KO males also had a significantly higher courtship index than control males towards mated females (Figure 8B).

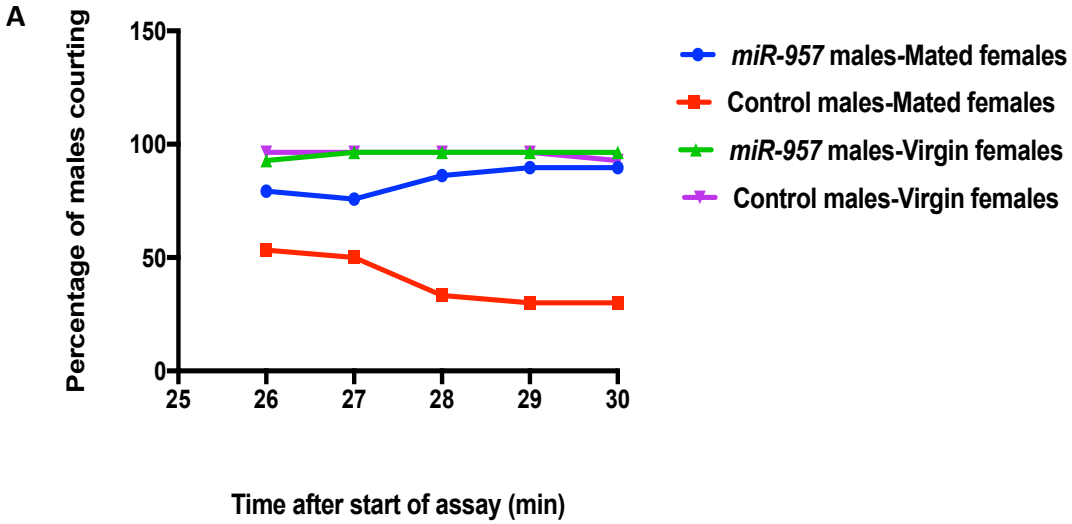

Figure 8. *miR-957* KO males reduce courtship efforts towards mated *D. melanogaster* females. A) The proportion of *miR-957* or control males that court decapitated *D. melanogaster* mated or virgin females during the last 5 min of a 30-min assay. **B)** CI of *miR-957* or control males towards decapitated *D. melanogaster* mated or virgin females during the last 10 min of a 30-min assay. CI values were compared using Mann-Whitney U-tests. N=28-30 for each genotype. Error bars represent mean ± standard error (SEM).

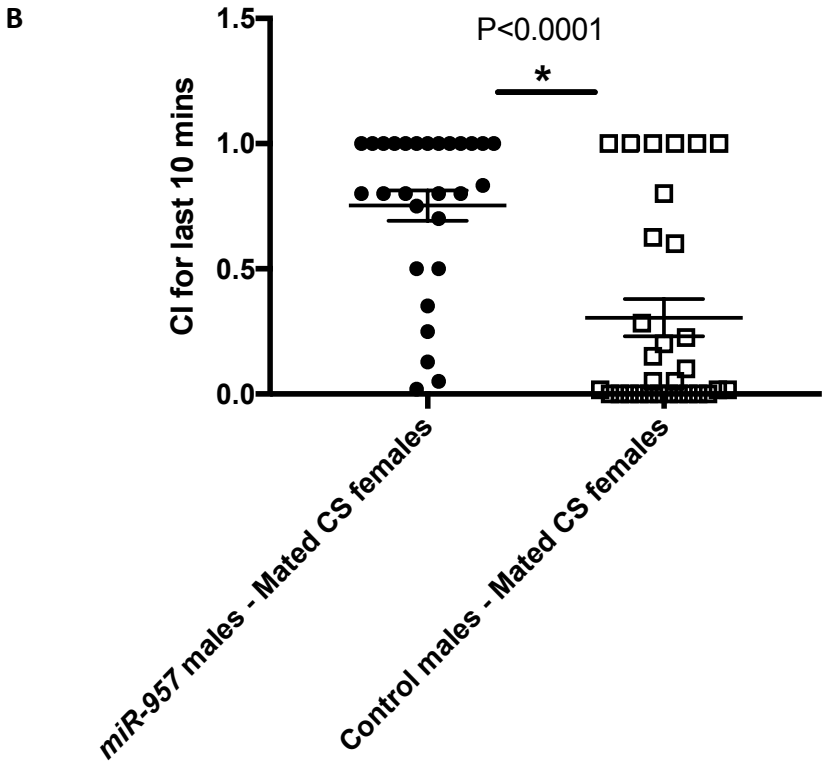

Figure 8. Continued

As expected, a low percentage of control males courted *D. simulans* females after a 25 min exposure to the heterospecific females. Compared to control males, roughly twice as many *miR-957* KO males continued to court *D. simulans* females after a 25 min exposure (Figure 9A). When we calculated the courtship index of males towards *D. simulans* females, we found that *miR-957* KO males had a significantly higher courtship index than control males (Figure 9B).

These results indicated that *miR-957* KO males may not perceive male-aversive pheromones correctly, which may account for their heightened courtship towards other males

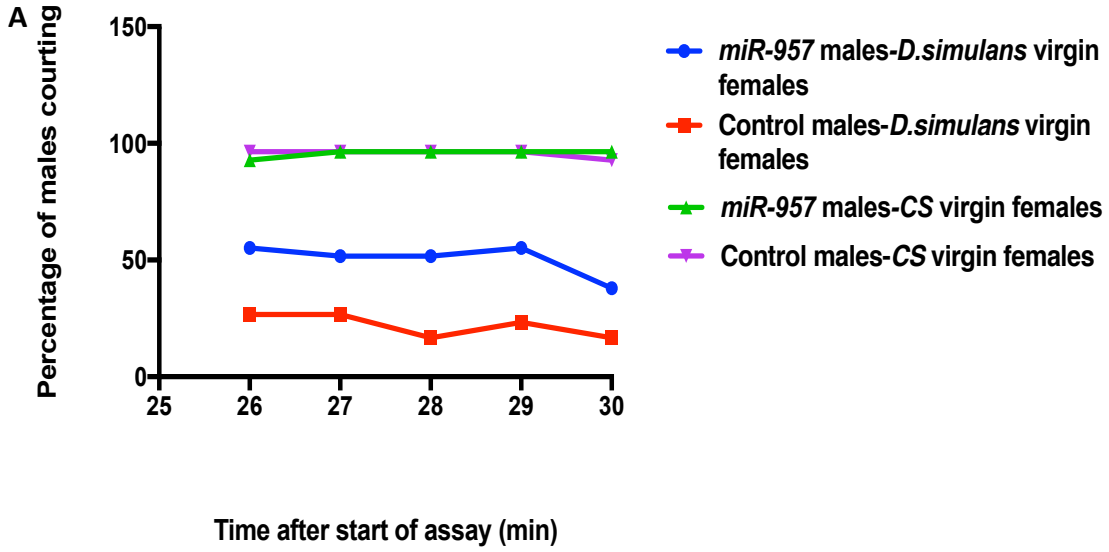

Figure 9. *miR-957* KO males reduce courtship efforts towards virgin *D. simulans* females. A) The proportion of *miR-957* or control males that court decapitated virgin *D. simulans* or *D. melanogaster* females during the last 5 min of a 30-min assay. **B)** CI of *miR-957* KO males or control males towards decapitated virgin *D.simulans* and *D. melanogaster* females during the last 10 min of a 30-min assay. CI values were compared using Mann-Whitney U-tests. N=28-30 for each genotype. Error bars represent mean ± standard error (SEM).

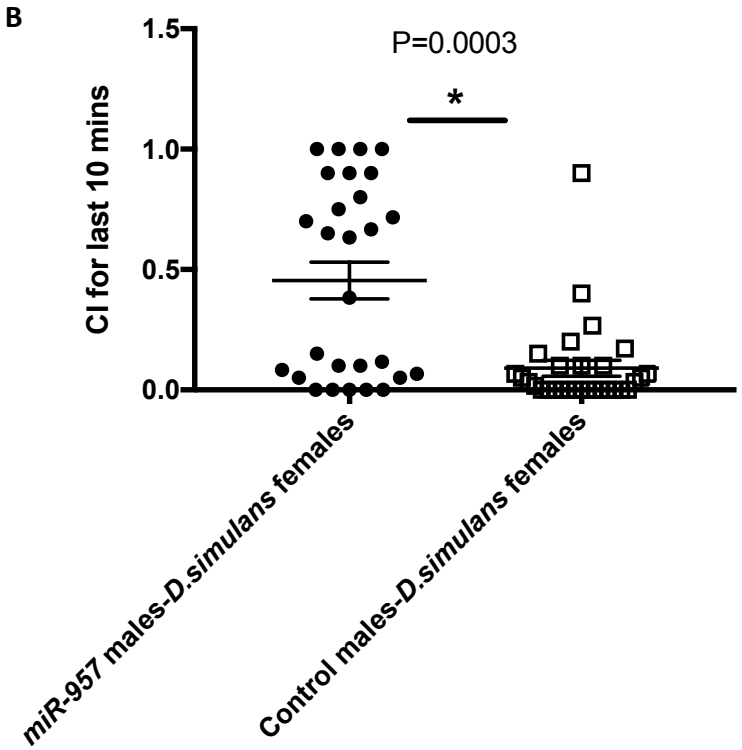

Figure 9. Continued

3.3.6 *miR-957* KO males are less responsive to 7-T and cVA

To identify the aversive pheromone(s) that *miR-957* KO males are less responsive to, we exposed these males to females perfumed with 7-T or cVA and measured the courtship index for the last 10 minutes of a 30-min assay (Figure 10A and 10B). When males were exposed to females coated with 7-T, the male genotype as well as the substance on the female had a significant effect on courtship index, and the interaction between the male genotype and substance perfumed was also significant (two-way ANOVA: Male

genotype × Female pheromone: F(1, 117) = 4.027; P=0.0471; Male genotype: F(1, 117) = 40.32; P < 0.0001; Female pheromone: F(1, 117) = 40.32; P<0.0001). These results indicate that differences in courtship index towards the different females are not consistent for both male genotypes. The perfuming of 7-T on females dramatically reduces the courtship index of control males (one-way ANOVA: F (3,118) = 22.01; P<0.0001Tukey's post-hoc test: control males with hexane-treated females vs. control males with 7-T treated females: P<0.0001, N_1=28, N_2=29), while *miR-957* KO males do not show a significant reduction in courtship index towards females perfumed with 7-T when compared to the courtship index of *miR-957* KO males towards hexane-treated females (P<0.2280, N_1=N_2=32) (Figure 10A). There was also a significant difference between courtship index of control males and *miR-957* KO males towards 7-T treated females (P<0.0001). We noted that *miR-957* KO males had higher courtship index towards hexane-treated females as compared to the courtship of control males towards hexane-treated females (P=0.0124). Regardless of the basal courtship towards hexane-treated females it is clear that control males show a very strong behavioral response to the addition of 7-T whereas the response is nearly absent in *miR-957* KO males.

For experiments with cVA-treated females, the interaction between the male genotype and substance perfumed was significant (two-way way ANOVA: Male genotype × Female pheromone: F (1, 111) = 3.964; P=0.0489; Male genotype: F(1, 111) = 18.23; P < 0.0001; Female pheromone: F(1, 111) = 35.63; P<0.0001). Again, the response to the substance perfumed was genotype dependent. Perfuming of cVA on females significantly lowered the courtship index of control males (one-way ANOVA: F

(3,111) = 20; P<0.0001; Tukey's post-hoc test: control males with ethanol-treated females vs. control males with cVA treated females: P<0.0001, N_1=31, N_2=32). Unlike when females were perfumed with 7-T, when females were perfumed with cVA *miR-957* KO males showed a reduction in courtship index towards females as compared to their courtship index towards ethanol-treated females (P<0.0364, N_1=N_2=27). Yet, there is a significant difference between the courtship index of control and *miR-957* KO males towards cVA treated females (P<0.0001), compared to the difference between the courtship index of control and *miR-957* KO males towards ethanol-treated females, which was not significant (P=0.3798). Although these males are responding to cVA, the response is much lower than the response observed from control males.

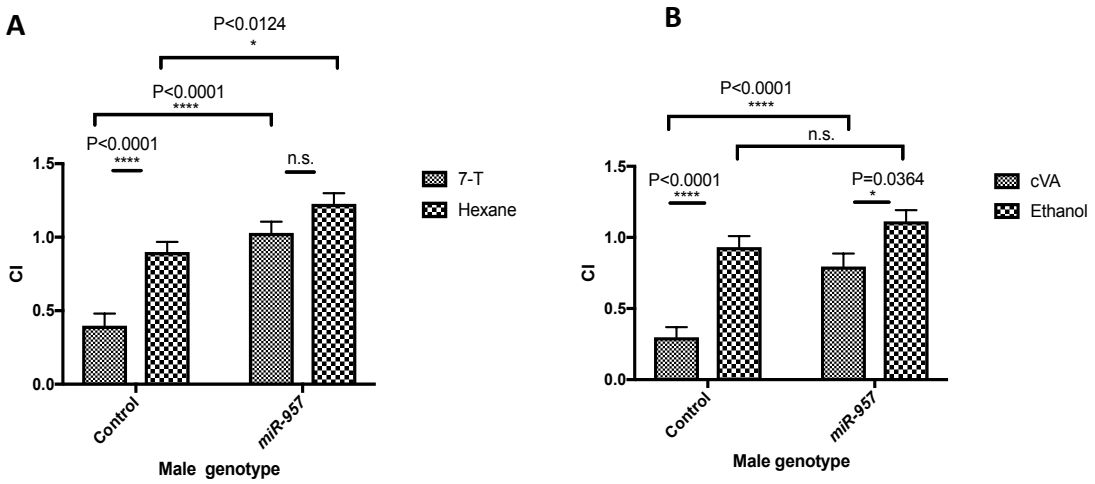

Figure 10. *miR-957* KO males show reduced inhibitory response to 7-T and cVA. A) CI of *miR-957* KO males or control males towards 7-T or hexane treated females during the last 10 min of a 30-min assay. N=28-32 for each genotype. **B)** CI of *miR-957* KO males or control males towards cVA or ethanol treated females during the last 10 min of a 30-min assay. N=27-31 for each genotype. CI values were compared using one-way ANOVA followed by post-hoc Tukey Kramer tests. Error bars represent mean ± standard error (SEM).

3.4 Discussion

3.4.1 *miR-957* KO males perceive male courtship-inhibiting pheromones differently and therefore display male-male courtship

miR-957 is a relatively unexplored miRNA that does not have mammalian orthologs but is conserved in four species of Drosophila and three species of mosquito (miRBase,

http://www.mirbase.org) (RUBY *et al.* 2007; STARK *et al.* 2007; KOZOMARA AND GRIFFITHS-JONES 2014). In our study, *ales*displayed high levels of inter-male courtship. These males did not elicit more courtship from wild-type males but courted males with significantly higher intensity. This result suggested that the pheromonal profile of *miR-957* KO males had not changed but, instead, that they failed to perceive sexual cues from courtship subjects correctly. *miR-957* KO males courted females at levels similar to the courtship displayed by control males, and they chose female subjects when given the choice to choose between males or females. These results indicated that their perception of female attractive pheromones was intact, but *miR-957* mutants failed to recognize males as futile targets for courtship. To determine if *miR-957* KO males are not receiving/processing male aversive cues correctly, we tested their courtship towards two types of female subjects, *D. simulans* females and mated *D. melanogaster* females, which are known to have elevated levels of pheromones that inhibit male courtship. We found that *miR-957* mutants did not reduce courtship towards mated *D. melanogaster* females or *D. simulans* females as fast as the control males, suggesting that *miR-957* KO males are responding differently to the bouquet of pheromones that are generally sexually aversive for *D. melanogaster* males.

3.4.2 *miR-957* is important for detecting pheromones, including 7-T and cVA, that contribute to chemical mate-guarding and for maintaining the sex and species-specific courtship barriers

Male-male courtship behavior observed in *miR-957* KO males could be due to altered perception of either a specific male-courtship inhibiting pheromone or due to a change in the processing of information from multiple male sexually aversive pheromones. Potential candidate pheromones include 7-T. This pheromone has been classified by most studies as a gustatory cue associated with male-male aggression and is well described as an aversive compound for male-male courtship (SAVARIT *et al.* 1999; BILLETER *et al.* 2009; WANG *et al.* 2011). 7-T is present in much higher quantities on *D. melanogaster* males compared to females (LATURNEY AND BILLETER 2016). Some studies have also suggested that it is only found on virgin males and is not present on virgin females (EVERAERTS *et al.* 2010). Genetic ablation of 7-T producing oenocytes results in an increase in male-male courtship, while application of 7-T onto these males rescues the phenotype (SAVARIT *et al.* 1999; BILLETER *et al.* 2009; WANG *et al.* 2011), suggesting a role for 7-T in providing a sex-specific barrier to courtship. Mutating one of the receptors for 7-T, GR32a, increases male courtship towards mated females (MIYAMOTO AND AMREIN 2008). During copulation Drosophila males deposit aversive pheromones onto females to prevent or delay copulations with other males, a phenomenon known as chemical mate guarding. The fact that loss of 7-T receptor causes males to court mated females suggests its additional role in chemical mate-guarding (MIYAMOTO AND AMREIN 2008).

Amongst the well-characterized volatile olfactory ligands is the male inhibitory pheromone cVA that is produced in the male ejaculatory bulb and is transferred to females during mating (BUTTERWORTH 1969; BRIEGER AND BUTTERWORTH 1970). cVA is also suspected to be important for reducing male-male courtship (EJIMA *et al.* 2007; WANG AND ANDERSON 2010). cVA appears to be another anti-aphrodisiac that is not being detected by *miR-957* KO males since the males continue to court females that have been perfumed with cVA. It is also plausible that the increased male-male courtship shown by *miR-957* KO males is due to an altered response to a combination of pheromones, including 7-T and cVA, and not to one specific pheromone. This possibility is in line with the finding that cVA and 7-T interact to reduce the attractiveness of mated females (LATURNEY AND BILLETER 2016).

Our perfuming experiments showed that *miR-957* KO males do not reduce their courtship significantly towards females perfumed with 7-T, whereas the control males do so within a 30-min assay. *miR-957* KO males also had higher courtship index towards hexane-treated females as compared to the courtship of control males towards hexane-treated females. Besides being defective for detecting 7-T, *miR-957* KO males may also be hypersensitive to an attractive pheromone present on the females. However, this scenario is unlikely because we did not observe a difference between the courtship of *miR-957* KO males and control males towards ethanol-treated females. Additionally, the difference between the courtship index of control and *miR-957* KO males towards 7-T treated females is more pronounced compared to the difference between their courtship index towards hexane-treated females. Interestingly, *miR-957* KO males reduce

courtship efforts in the presence of cVA, although the response is weaker than the response shown by control males. These findings make sense in light of the emerging understanding that 7-T plays a dominant role in preventing male-male courtship as well as inter-specific mating, and in our study *miR-957* KO males that were less responsive to 7-T were observed to display both male-male courtship as well as very high heterospecific courtship. 7-T is one of the compounds that is transferred onto females during copulation and stays on them much longer than most seminally transferred compounds (YEW *et al.* 2009; EVERAERTS *et al.* 2010). cVA, on the other hand, is now viewed as a regulator of response to other pheromones by increasing or decreasing repulsion that is already there to modulate the intensity of specific social behaviors (BILLETER AND LEVINE 2015; BILLETER AND WOLFNER 2018). cVA must be sensed in conjunction with 7-T in order to influence aggression (WANG *et al.* 2011) and courtship behavior (LATURNEY AND BILLETER 2016).

Differences in the response to 7-T and cVA could arise through a variety of mechanisms. For example, the sensory pathway for the two pheromones differs. cVA is detected primarily by the olfactory system by a specialized trichoid sensillum, while the putative receptor for 7-T is a gustatory receptor, Gr32a (MIYAMOTO AND AMREIN 2008; WANG *et al.* 2011; LATURNEY AND BILLETER 2016). For *miR-957* to be involved in the detection of both cVA and 7-T, it would need to be expressed in both gustatory and olfactory sensing channels. Expression of *miR-957* mainly in the gustatory pathways can explain the stronger impact on 7-T perception and a lesser one on the response to cVA. Apart from the requirement of *miR-957* in sensing pathways, its possible expression in

higher order neuronal connections from neurons delivering aversive gustatory input could also explain the requirement of the miRNA in 7-T perception.

3.4.3 *miR-957* expression is needed in *dsx*-expressing and mushroom body (MB) neurons to prevent misdirected courtship

Our experiments with the *miR-957* sponge showed that *miR-957* expression is needed in *dsx*-expressing neurons for inhibition of male-male behavior. DSX transcription factors are essential for cell sexual identity as well as sex-specific behaviors and secondary sexual characteristics. In males, *dsx* is alternatively spliced to produce male specific DSX^M. Expression of DSX^M is not only necessary for proper display of male courtship steps, but studies have shown it to be important for the perception of sensory cues needed for appropriate mate recognition (REZAVAL *et al.* 2016). Surprisingly, we did not observe any male-male courtship when the sponge was expressed in *fru* neurons. There is a partial overlap in the neurons that express *fru* and *dsx*. In particular, the majority of brain and thoracic ganglion neurons expressing *dsx* also express fru^M. However, there are several neurons that express only *dsx* and are known to be sufficient to induce male courtship behavior. These include neurons in the PC1 cluster (REZAVAL *et al.* 2016). The observations from the sponge experiments thus suggested that the subfraction of *dsx*-expressing neurons that do not express *fru* is important for appropriate mate recognition. Another possibility is that *miR-957* expression is needed in all *dsx* neurons and not only in the fraction that overlaps with *fru* neurons to allow correct mate recognition.

We also detected male-male courtship behavior upon suppressing *miR-957* expression in the MB, which is a structure implicated in learning and olfactory processing (KIM *et al.* 2007) as well as mate discrimination (FERVEUR *et al.* 1995). It has been shown that the feminization of the MB via expression of *tra* induces bisexual behavior in males. Since MB is also need for olfactory processing it was speculated that MB is a structure important for the analysis of sex-specific pheromones (FERVEUR *et al.* 1995). *miR-957* could have a role in the MB in this context. Alternatively, it may be that *miR-957*-expressing MB neurons may communicate with higher order *dsx* neurons in the ventral nerve cord (VNC). Overall the results suggest that male-male courtship in *miR-957* KO males is potentially a result of altered pheromone processing, more likely at the level of integration of sexually aversive cues in the higher order processing centers such as the MB.

4. EXPRESSION OF miRNAs IN DROSOPHILA FAT BODY AND HEMOLYMPH

4.1 Introduction

In order to maintain a steady physiological state under varying environmental conditions, communication needs to be maintained among different organ systems. Individual organs can act as 'sensors' of fluctuations in conditions such as temperature and nutritional state and then relay this information to other organs, which bring about the appropriate homeostatic response.

Metabolic homeostatic responses are critical for organismal adaptation as they link the state of food reserves of an organism with caloric intake. Vertebrate adipose tissue acts as one of the sensors needed to relay information about circulating and stored nutrients to the central nervous system (CNS), which then sends signals to peripheral organs to adjust energy expenditure. One well-known example of a protein secreted by the mammalian adipose tissue to bring about such an effect is Leptin. Leptin is released by the adipose tissue to signal satiety to the hypothalamus under fed conditions so that a drop in energy expenditure and feeding occurs (ZHANG *et al.* 1994).

Such communication between organs can be studied more conveniently in a model organism that has an open circulatory system such as *Drosophila melanogaster*. Instead of complex organs such as liver and pancreas, Drosophila has a cluster of cells and tissues that are functionally analogous to their vertebrate counterparts. The insect fat body is considered the functional equivalent of vertebrate adipose tissue because it

serves as a repository for lipid and glycogen stores, but it also is an endocrine gland that produces and releases factors into the hemolymph (fly plasma). Several studies have demonstrated that the fat body in *Drosophila melanogaster* can also be involved in long distance signaling (RAJAN AND PERRIMON 2011). Particularly, it has been proposed that fat body secreted factors are needed for release of *Drosophila* insulin like peptides (Dilps) from a cluster of neurosecretory cells, the insulin-producing cells (IPCs) (GEMINARD *et al.* 2009; RAJAN AND PERRIMON 2012).

There are several pieces of evidence demonstrating cross-talk between fat body and CNS. Firstly, the fat body communicates amino acid levels to the CNS. Reducing fat body levels of the amino acid transporter *slimfast (slif)* results in growth defects similar to growth defects observed upon blocking insulin secretion from the brain IPCs (COLOMBANI *et al.* 2003; RAJAN AND PERRIMON 2012). Moreover, the *Drosophila* cytokine Unpaired2 (Upd2) is produced in the fat body in response to lipid and sugar intake. When secreted by the fat body, Upd2 activates JAK/STAT signaling in a population of GABAergic neurons that project onto the insulin-producing cells, resulting in the secretion of Dilps into the hemolymph. Since Upd2 is secreted in response to sugar and lipid intake, but not changes in amino acid levels, it is likely that other unidentified factors also contribute to the coupling of nutritional status and Dilp secretion (RAJAN AND PERRIMON 2012).

Besides metabolic regulation, the fat body has also been shown to affect courtship behavior through the secretion of proteins. Lazareva et al. (2007) found that feminization of adult fat body resulted in a decrease in the courtship behavior of males

(LAZAREVA *et al.* 2007). They demonstrated that the male-specific, fat body expressed protein Takeout is secreted into the hemolymph and is needed for optimal mating.

It is now known that several tissues mediate extracellular communication by actively secreting microRNAs (miRNAs), which are 19-23 nucleotide (nt) non-coding RNAs that mostly act as translational inhibitors by binding to the 3'UTR of their target mRNAs (BARTEL 2009). Strikingly, miRNAs were shown to be present in wide array of biological fluids (WEBER *et al.* 2010). miRNAs are found in circulation primarily in extracellular vesicles (EVs) (PEGTEL *et al.* 2010; TKACH AND THERY 2016) or in association with proteins that protect them from degradation by RNases (VICKERS *et al.* 2011).

While most studies on circulating miRNAs have emphasized classifying miRNAs as biomarkers for cancers and other diseases, fewer have focused on understanding if these miRNAs can be exchanged between tissues and have functional relevance. Previously, the greatest limitation to answering these questions was the unavailability of methods that could be used to study long range signaling of miRNAs. While tagging miRNAs and tracking them in circulation remains a challenge, in the past few years strategies have been developed to tag EVs that carry non-coding RNAs in circulation and also fluorescently label the mRNA content of these vesicles. Furthermore, Lai et al. (2014) fused luciferase protein to a vesicular, membrane domain protein and measured luciferase activity in distant cells to demonstrate exchange of EVs. They also designed an intracellular probe to fluorescently label the mRNA content of the

EVs (LAI *et al.* 2015). Although these tools are promising, so far they have been used to demonstrate only in vitro vesicle-borne mRNA exchange between cultured cells.

A more promising methodology for in vivo studies has been expressing CRE recombinase in specific cells and quantifying recombination in recipient populations. Ridder et al. (2014) showed that CRE mRNA but not protein, was present in the EVs of mice expressing CRE in immune cells. These animals could also express a LacZ reporter only upon recombination-mediated excision of a STOP cassette. Strikingly, some non-immune cells, including certain neurons showed LacZ expression. The group controlled for effects of leaky expression of immune-cell promoter and fusion of cells containing CRE and LacZ reporter. Even though the CRE-mediated recombination efficiency was low and the nature of EVs was not identified, the tool is promising for understanding the nature of EV content exchanged between cells in vivo (RIDDER *et al.* 2014). Similarly, Zomer et al. (2015) used the CRE system to convert DsRed+ cells to eGFP+ cells and visualize them using intravital imaging (ZOMER *et al.* 2015). This study, like that of Ridder et al. (2015) did not identify the mechanism of vesicular uptake by recipient cells. The exact nature of the contents of the vesicular carriers was also not discussed.

Recent work is elucidating mechanisms for the amplification and spread of non-coding RNAs in Drosophila via exosomes. Exosomes are endosome-derived vesicles, less than 150nm in diameter. In Drosophila, hemocyte derived exosomes were shown to be carriers of viral small interfering RNAs (vsRNAs). Small interfering RNAs (siRNAs) guide RNA silencing complexes to endonucleolytically cleave viral transcripts. In the study by Tassetto et al. (2017), hemocytes took up viral RNA from infected tissues and,

using endogenous transposon reverse transcriptases, produced virus-derived cDNA. Using the cDNA template vsRNAs were synthesized *de novo* and packaged and secreted into exosomes. Remarkably injecting these exosomes along with a Drosophila virus into naïve flies could confer virus-specific immunity in the flies, suggesting that exosomes were taken up by uninfected tissues to provide systemic antiviral immunity (TASSETTO *et al.* 2017). The limitation of this study however, was that uptake of exosomes carrying svRNA was not visualized.

Although the importance of miRNAs in major Drosophila organs (discussed below) is now understood, there is a need to develop methods to understand if these miRNAs are exchanged between tissues. Several major Drosophila organs communicate via exchange of factors in the Drosophila's open circulatory system, yet it remains to be understood if non-coding RNAs such as miRNAs play an important role in this signaling.

4.1.1 Background: The role of miRNAs in the fat body

Some of the most intriguing examples of miRNAs that are part of homeostatic mechanisms come from the studies of miRNAs expressed in the fat body (XU *et al.* 2003; TELEMAN *et al.* 2006; HYUN *et al.* 2009; JIN *et al.* 2012; ESSLINGER *et al.* 2013; BARRIO *et al.* 2014). A sophisticated study that demonstrated the role of fat body expressed miRNAs in feeding behavior and metabolism was carried out by Vodala et al. (2012). The study first identified cycling miRNAs via Illumina Deep Sequencing of 18-29 nt RNAs isolated from the Drosophila head which is comprised of cuticle with

external sensory structures that encapsulates the brain-associated fat body and brain. The *miR-959-964* cluster, a head fat body-expressed miRNA with robust rhythmic expression, was shown to be involved in the regulation of timing of feeding behavior. Intriguingly, feeding, in turn, appeared to control the temporal aspect of the cluster's transcription (VODALA *et al.* 2012).

More recently a miRNA overexpression screen implicated *miR-iab-4* as a fat body-expressed regulator of feeding (Kim et al. 2017). Although the authors found some miRNAs in their primary screen that reduced feeding or enhanced feeding by around 35%, none of the individual miRNAs had a statistically significant impact on feeding. *miR-iab-4* overexpression flies consumed 27% more food than wildtype flies. The effect of *miR-iab-4* overexpression was also tested with a different fat body driver from the one used previously and the effect was confirmed. The authors then carried out a secondary RNAi screen for predicted targets of *miR-iab-4.* The rationale behind the secondary screen was that reducing the expression of a *miR-iab-4* target would likely produce a stronger effect. The secondary screen showed that reduction of *purple,* a predicted target of *miR-iab-4,* increased the feeding activity of the flies. *Purple* is involved in synthesis of an intermediate product 6-pyruvoyltetrahydropterin (PTP). PTP appears to circulate to the brain where it is converted to tetrahydrobiopterin BH4. This conversion appeared to be regulated in neurons producing the neuropeptide NPF, a protein previously associated with feeding. The effect of *purple* on feeding behavior was maintained even when purple levels were reduced in adults only. However, the study did not demonstrate that *purple* expression is regulated by *miR-iab-4.*

The work of Barrio et al. (2014) is also in line with the findings that fat body-expressed miRNAs can enable nutrient sensing. They showed that fat body-expressed *miR-305* regulates expression of Dp53, the fly homolog of p53. Barrio et al. (2014) identified a role for Dp53 in nutritional homeostasis in flies whereby Dp53 slows the breakdown of energy stores under conditions of nutrient deprivation. The protein was shown to be regulated by *miR-305*. In fact, the group demonstrated that under conditions of nutrient deprivation there was an overall reduction in miRNA biogenesis in the fat body, which led to elevated levels of Dp53 and a reduced rate of nutrient break down. These findings were a remarkable demonstration of how miRNAs can be important for adaptive responses to environmental changes, a role that has frequently been associated with miRNAs (BARRIO *et al.* 2014; HE *et al.* 2016).

4.1.2 Background: The role of miRNAs in the brain

The role of miRNAs in learning and memory was first suggested by Li et al. (2013) through a study that demonstrated that *miR-276a* tuned the expression of dopamine receptor in mushroom body neurons for memory formation and in ellipsoid body for naïve responses to odors. The expression of a miRNA sponge construct regulated by neuronal promoters and temporally controlled by GAL80 allowed testing for the post-developmental function of *miR-276a* (LI et *al. 2013)*. The results demonstrated that the phenotype was an adult phenotype not a developmental one. One of the important ideas that emerged from this study was that even within one organ, miRNAs can have diverse functions in different brain compartments based on the mRNAs targeted in the different

areas. A much larger survey for miRNA function in memory formation utilized miRNA sponges and identified 5 different miRNAs (*miR-9c*, *miR-31a*, *miR-305a*, *miR-974* and *miR-980*) that affect memory formation (BUSTO *et al.* 2015). This screen showed that *miR-980,* unlike *miR-276a,* had a shared rather than compartmentalized effect across several neuronal clusters that led to enhanced memory.

While most recent miRNA studies have looked at the effects of reducing miRNA levels, Bhat et al. (2015) overexpressed miRNAs in adult olfactory neurons to identify miRNAs that modify expression of the odorant receptor Or47b. For all the miRNAs that modified levels of Or47b, predicted targets were listed and targets that were common among the miRNAs that reduce Or74b expression were identified. A secondary RNAi screen for targets was then performed with the expectation that reduction of the target mRNA levels should phenocopy the effects of miRNA overexpression. Through this strategy, Atf3 was implicated in regulating Or47b expression (Bhat et al. 2015).

Another context in which miRNA functions have been explored in the fly brain is the post-transcriptional regulation of the circadian clock. Yang et al. (2008) did pioneering work in identifying a set of miRNAs under circadian regulation, of which *miR-263a* and *miR-263b* were predicted to target core clock proteins. Kadener et al. (2009) further demonstrated that reducing miRNA biogenesis in circadian oscillator cells resulted in aberrant behavioral rhythms and increased transcription of several transcripts under circadian control. Interestingly, although most circadian research has focused on the neuronal clock, glial cells, which support neuronal activity, also have been shown to be contributors to rhythmic behavior. Recently, a genome-wide screen for glial miRNAs

that influence circadian behavior was published by You et al. (2018) using miRNA sponges to specifically reduce glial miRNA levels. Whereas 20 glial miRNAs affected circadian behavior, only two were investigated further. *miR-263b* and *miR-274* were found to have circadian functions in adult flies, although no targets for *miR-263b* were identified with genetic approaches. The decreased rhythmicity observed in the mutants for these two miRNAs was attributed to post-developmental defects after it was confirmed that there were no obvious morphological differences between experimental and control neurons. Adult-specific manipulation *miR-274* expression resulted in the same phenotype as the flies with constitutively expressed sponges, further supporting a post-developmental role for *miR-274* in rhythmicity. For both *miR-263b* and *miR-274,* since overexpression of the miRNA also decreased rhythmicity, it was suggested that reducing expression of its mRNA target would phenocopy the effect. Therefore, the researchers performed RNAi-based screens on in silico predicted targets of the two miRNAs. While no targets were validated for *miR-263b,* two were confirmed for *miR-274*, both of which have mammalian orthologs.

miRNAs thus play important roles in major Drosophila organs such as brain and fat body. In view of the fact that remote control of neurobiological processes by fat body secreted factors has been demonstrated (GEMINARD *et al.* 2009; RAJAN AND PERRIMON 2012) and that miRNAs are an emerging class of factors released by the adipose tissue (OGAWA *et al.* 2010; MULLER *et al.* 2011), I hypothesized that apart from the exchange of proteins, the exchange of genetic information in the form of miRNAs is also needed for cross-talk between adipose and neural tissues. At the time we started this study, there

was no literature indicating that miRNAs are present in circulation in insects and could be exchanged between tissues. In order to test my hypothesis I started by testing for the presence of miRNAs in hemolymph. In the following section I describe experiments I carried out to determine if miRNAs expressed in the fat body only act cell-autonomously or if they can have systemic effects as a result of non-cell autonomous actions. I will also discuss the problems encountered in the process and propose potential future directions.

4.2 Methods

4.2.1 Fly stocks

Fat body drivers *cg-gal4* (BDSC#7011) and *r4-gal4* (BDSC# 33832) and the *dicer-1* RNAi line, *UAS-dcr-1RNAi* (BDSC#34826), were obtained from the Bloomington Drosophila Stock Center. Flies carrying a *UAS-CD63-GFP* were gifted by Clive Wilson.

4.2.2 Scoring courtship index (CI) of flies with *dicer-1* (*dcr-1*) reduction in the fat body

Single pair mating assays were conducted (as described in chapter 2) with males of the following genotypes: *cg-gal4>UAS-dcr-1RNAi* and *r4-gal4> UAS-dcr-1RNAi* (experimental flies), *cg-gal4* and *r4-gal4* (drivers) and *UAS-dcr-1RNAi* (reporter). CI was calculated as the proportion of time spent courting by a male during the first 10 min of exposure to a female subject.

4.2.3 Hemolymph isolation

10-12 third instar wandering larvae were dipped in DMPC treated phosphate-buffered saline (PBS) to remove food particles, followed by dipping in 95% ethanol for surface sterilization. They were quickly transferred to a filter paper to remove excess ethanol. The larvae were pinched close to the mouth hooks with a pair of forceps and transferred to a 0.5ml Eppendorf with an approximately 0.5 inch slit at the base. This tube was then placed in a 1.5ml Eppendorf and centrifuged for 5 min at 3000g. A tiny droplet (~2-3 ul) of hemolymph was collected at the base of the Eppendorf. 5ul of anti-coagulant Ringer's solution was added to the collected hemolymph and the mix was centrifuged again for 5 min at 3000g. The supernatant was then transferred to a new Eppendorf while making sure to leave the debris behind. The collected hemolymph droplet was quickly frozen using dry ice and transferred to a -80°C freezer. Before proceeding with miRNA/exosome isolation, hemolymph aliquots were thawed and pooled to yield a volume of approximately 500ul of the mix of hemolymph and Ringer's solution. The volume was adjusted to 1mL using RNAse free water. The mix was centrifuged through a 0.22um filter at 12000g.

4.2.4 Small RNA isolation

Small RNA isolation from the hemolymph was carried out using miRCURY RNA Isolation Kit – Biofluids (Exiqon) since ultracentrifugation techniques are not feasible with the small volumes of Drosophila hemolymph. To ensure that hemolymph samples were not contaminated with cells and that accumulation of miRNAs due to rupturing of

hemocytes (cellular components of hemolymph) was minimized, the hemolymph samples were mixed with Drosophila anticoagulant Ringer's solution. The samples were centrifuged before storage, and once the aliquots were pooled, the sample was passed through a 0.22um filter column to ensure removal of remaining cells, debris and large exosomal vesicles. miRNAs were isolated from three different fractions of hemolymph: 1) cell-free hemolymph, 2) exosomes isolated from cell-free hemolymph and 3) cell and exosome-free fraction of hemolymph.

miRNA isolation from fat body was conducted using the Ambion mirVana miRNA isolation kit was as per the manufacturer protocol.

4.2.5 Exosome isolation

Exosomes were isolated using miRCURY Exosome Isolation Kit - Cells, urine and CSF (Exiqon). Afterwards, small RNAs were isolated from the exosomes using Exiqon's miRCURY RNA isolation kit – Biofluids.

4.2.6 miRNA reverse transcription and cDNA amplification

For reverse transcription of miRNAs, the TaqMan miRNA Reverse transcription kit (5366596) was used along with stem-loop reverse transcription primers. These are modified primers designed to amplify miRNAs since miRNAs cannot be amplified with regular primers due to their small size and the absence of a polyA tail (KRAMER 2011). The manufacturer's protocol was used to set up 15ul reactions. TaqMan MicroRNA

Assays (4366597) were then used to amplify the reverse transcription product. Amplified product was visualized on a 4% ethidium bromide gel.

4.2.7 Ex vivo fat body culture

An ex vivo fat body culture experiment was conducted to determine if miRNAs could be released by cultured fat bodies into medium. Fat body was dissected from surface-sterilized 3rd instar wandering larvae, washed in PBS, and cultured in serum-free Schneider's medium for 24 hrs. miRNAs were isolated from filtered Schneider's medium and amplified using the method described previously for isolating and amplifying miRNAs from hemolymph samples.

4.2.8 Incubation of brains with fat body secreted, stained exosomes

Exosomes isolated from fat body culture experiments were labeled using SYTO RNA Select (Invitrogen) which preferentially stains RNA. Stained exosomes were incubated with brains for 3 hrs at 37 °C and then examined at 100x magnification, using confocal microscopy, for the uptake of exosomes and compared with a dye-only control.

4.2.9 Statistics

Statistical analysis for behavior was done using GraphPad Prism version 7.00 for MAC OS X, GraphPad Software, La Jolla California USA, www.graphpad.com. Values for courtship index were arcsine transformed and compared using a non-parametric Kruskal-

Wallis test (since the test for normality failed) followed by a post-hoc Dunn's test for multiple comparisons.

4.3 Results

4.3.1 Expression of *dicer-1* RNAi in the fat body results in reduced courtship index

Dicer-1 is one of the core proteins involved in miRNA biogenesis (Lee et al. 2003). We detected a significant reduction in CI when *dicer-1* (*dcr-1*) was reduced in the fat body using each of two different fat body drivers, *cg-gal4>dicer-1* RNAi (N=30) vs. *cg-gal4* (N=23) ($P<0.0001$), *cg-gal4>dicer-1* RNAi (N=30) compared to *dicer-1* RNAi (N=37) ($P<0.0001$) and *r4-gal4>dicer-1* RNAi (N=34) vs. *r4-gal4* (N=29) ($P<0.0001$), *r4-gal4>dicer-1* RNAi (N=34) compared to *dicer-1* RNAi (N=37) ($P<0.0082$) (Figure 11). Flies with fat body specific *dcr-1* reduction are not sick and, in fact, have higher survival rates than wildtype flies (Barrio et al. 2014). Therefore reducing miRNA biogenesis in the fat body, with two different fat body specific drivers, reduced courtship.

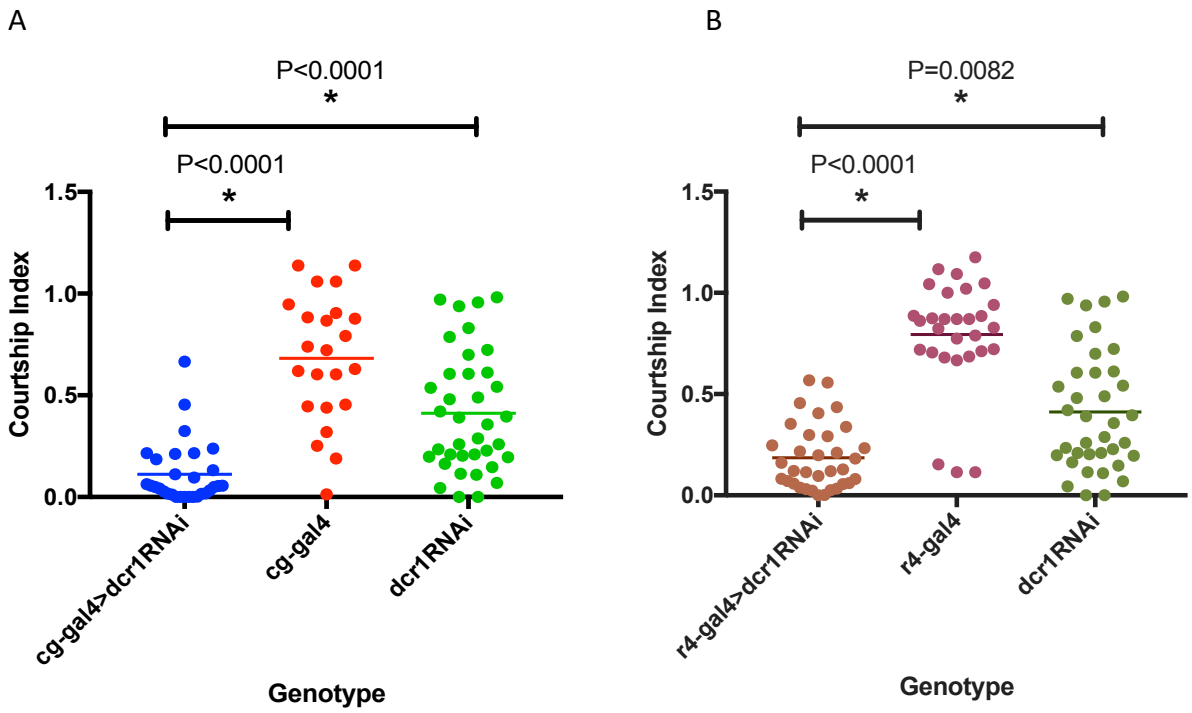

Figure 11. Males with decreased *dicer-1* (*dcr-1*) expression have reduced courtship index (CI). CI of males expressing *dcr-1* RNAi using either *cg-gal4* A) or *r4-gal4* B), N=23-37 for each genotype. CI values were compared using Kruskal-Wallis test followed by post-hoc Dunn's test. Bars represent the mean.

4.3.2 miRNAs are present in the hemolymph

The results described above suggested that blocking miRNA production in the fat body affects courtship behavior. This effect could be a systemic response to reducing miRNA biogenesis in fat body or an effect observed due to blockage of miRNA-mediated

communication between fat body and other tissues. To investigate the latter, we first tested for the presence of miRNAs in the hemolymph.

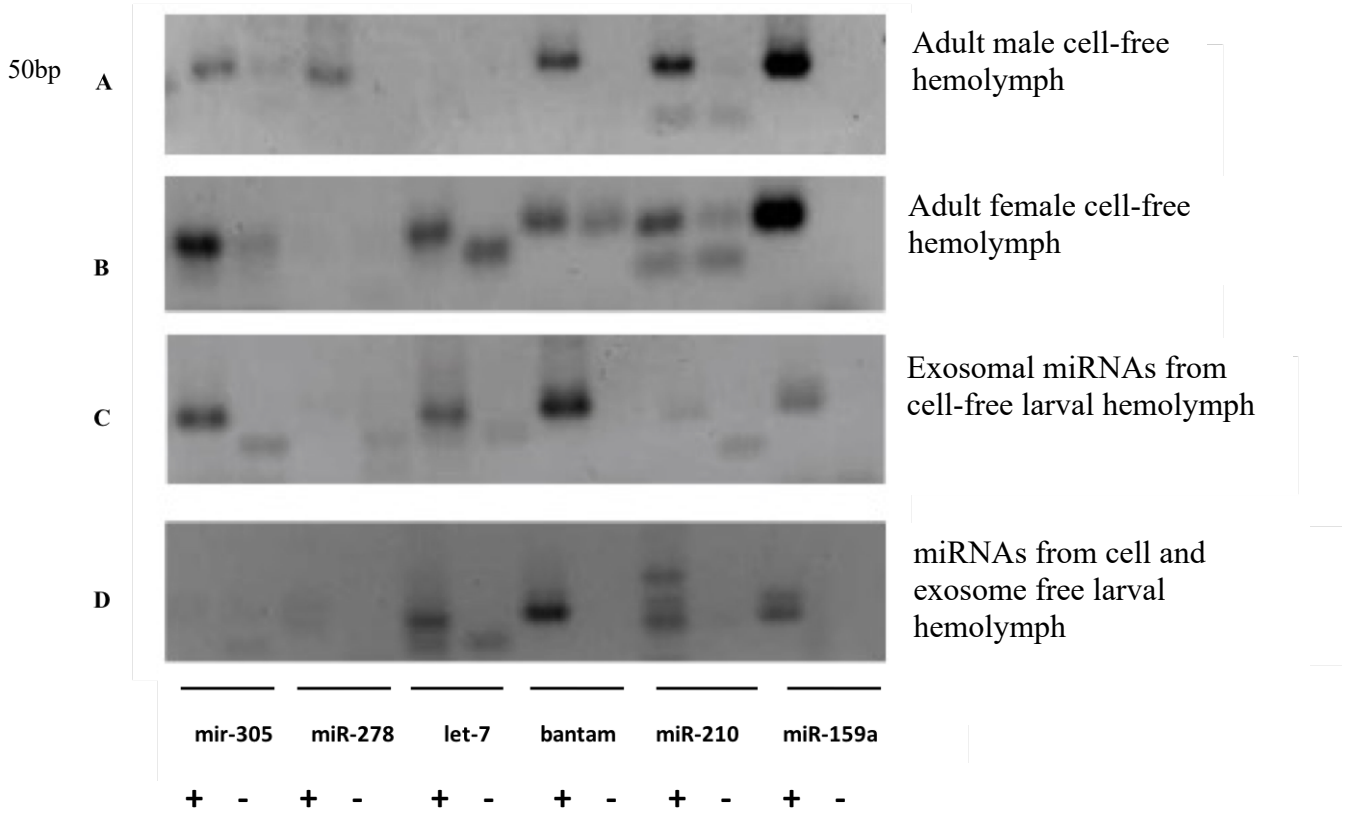

Figure 12: Detection of miRNAs in larval and adult hemolymph. Products of PCR amplification of cDNA obtained after miRNA-specific reverse transcription of RNA extracted from specified tissues. (+) indicates the addition of reverse transcriptase (RT) for cDNA production. (-) indicates the no RT control. *miR-159a* is an exogenous control.

We identified miRNAs in various fractions of the hemolymph (cell-free hemolymph (Figure 12 A and B), hemolymph exosomes (Figure 12 C), and cell and exosome-free hemolymph (Figure 12 D), thus confirming that miRNAs are present in circulation in Drosophila hemolymph (Figure 12). Exosomes are one of the major carriers of circulating miRNAs (Pegtel et al. 2010, Tkach et al. 2016) that are released by tissues in a regulated fashion. *miR-305*, *let-7*, *bantam* and *miR-210* were amongst exosomal small RNAs isolated from the larval cell-free hemolymph. *miR-305* was found only in the exosomal fraction of larval hemolymph. *miR-210* appeared to be present in lower amounts in isolated larval exosomes compared to hemolymph. We also observed sexually dimorphic expression of miRNAs in adult cell-free hemolymph (Figure 12).

4.3.3 Cultured fat body can release miRNAs

To test whether fat body could be a contributor to the miRNAs in the hemolymph, we tested if larval fat body can release miRNAs when cultured in Schneider's medium. *miR-278, let-7, bantam* and *miR-210* were detected in the Schneider's medium in which fat body was cultured (Figure 13). All of these miRNAs were detected in the fat body except for *miR-210.* This may be due to the higher amount of the primary miRNA transcript for *miR-210* within the fat body rather than the mature transcript that is predominantly secreted (mature transcript is the form detected by our primers). *miR-305* was detected when miRNAs were enriched from exosomes, but not in cell and exosome free hemolymph. This might be because the expression of *miR-305* only becomes detectable following enrichment from exosomes and is too dilute to be detected

otherwise. *let-7* and *bantam* were also enriched in the exosomal fraction. *miR-278* was detected in cell-free and exosome-depleted Schneider's medium but was absent from the exosomal fraction. miRNAs detected in the Schneider's medium may not be secreted through a regulated mechanism of release via exosomes but may be present as a result of cell death or apoptosis. However, prior ex vivo organ culture experiments demonstrated that cultured organs were physiologically stable for up to 4 days (AWAD AND TRUMAN 1997). Images of fat body after 20 or 60 hrs of culture showed that fat body maintains integrity at these time points (Figure 14).

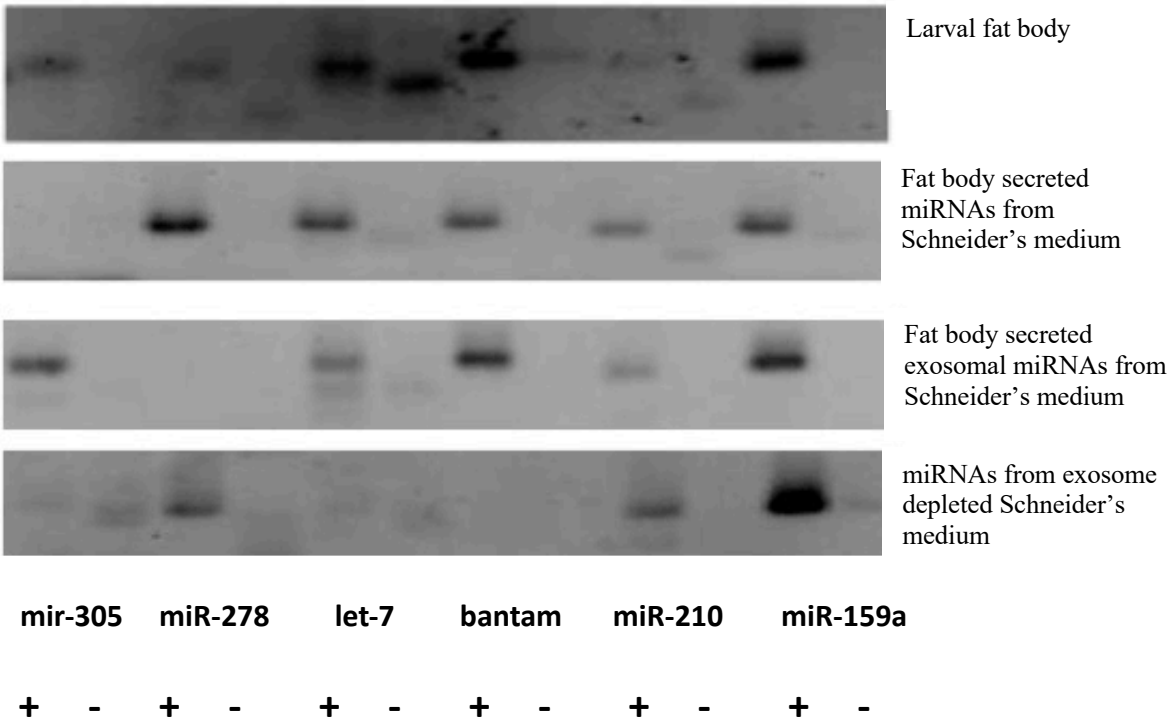

Figure 13: Detection of miRNAs in fat body and fat body culture medium after 24 hours. Products of PCR amplification using miRNA-specific primers. *miR-159a* is an exogenous control. (+) indicates the addition of reverse transcriptase (RT) for cDNA generation. (-) indicates the no RT control.

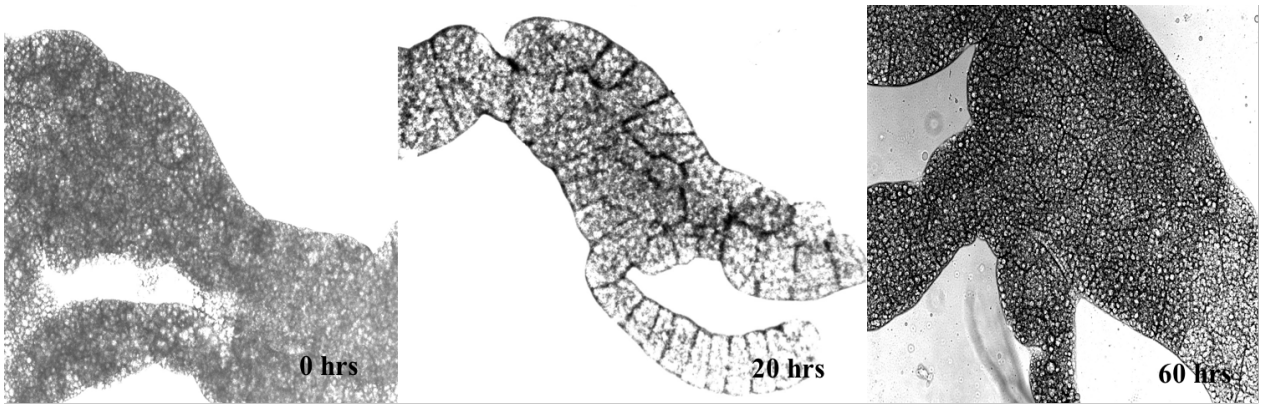

Figure 14: Fat body incubated in Schneider's medium. Fat body maintains integrity for at least 60 hrs when incubated in serum-free Schneider's medium. Times displayed in the lower panel indicate the number of hours of fat body culture. Images taken at 10x using a bright field microscope.

4.3.4 Fat body secreted exosomes can be taken up by the CNS in vitro

We incubated larval brains with exosomes that were collected from fat body cultures and stained them with an RNA-specific dye. Puncta were observed on the surface of and also within the brains incubated with SYTO RNA Select labeled exosomes when z-stacks of the brains were examined using a confocal microscope (Figure 15). These puncta were not observed in the dye-only controls. It is unlikely that the puncta indicate individual exosomes due to variability in their size. Instead, they are likely to be exosomes aggregates. Our results suggest that the exogenously supplied exosomes were up taken

by the CNS since the puncta were observed not only at the surface but also within the deeper sections of the experimental brains but not in the dye-only controls.

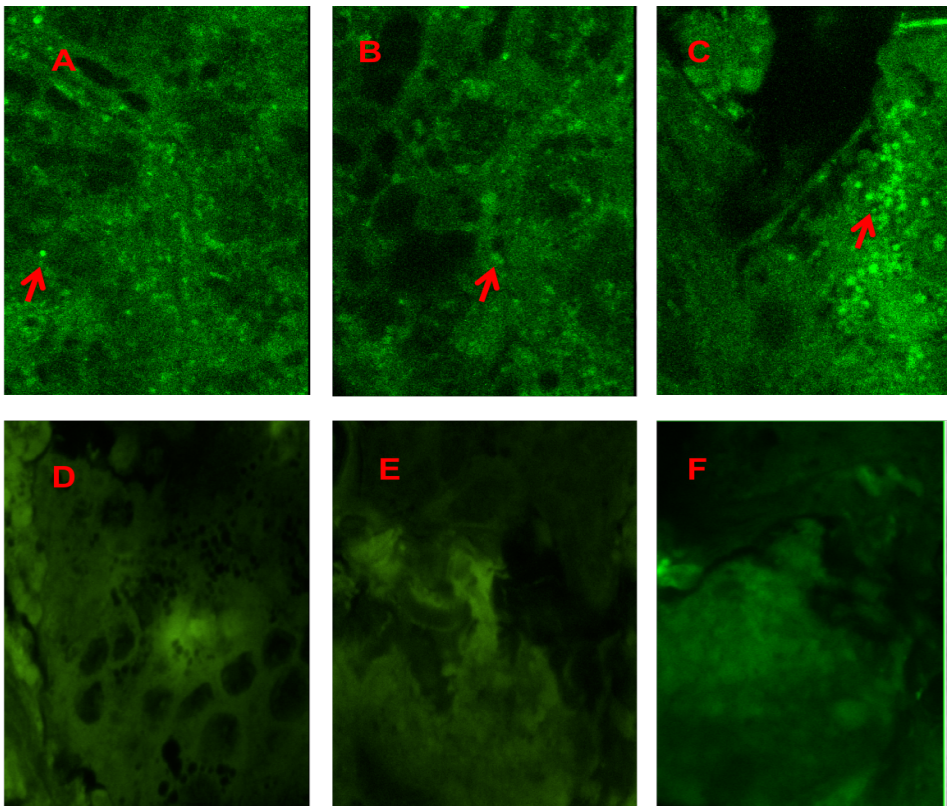

Figure 15: Brains incubated with exosomes labeled with SYTO RNA Select stain. A, B and C show brains incubated with labeled exosomes. D, E and F show the dye-only controls. Arrows point to labeled puncta that may indicate aggregates of exosomes. Images taken at 100x using a confocal microscope.

4.3.5 Reducing fat body exosome secretion to evaluate effects on courtship

To understand whether fat body secreted exosomes can communicate with other tissues, we also incorporated GFP-labelled CD63 in fat body exosomal membranes as they are produced using flies that express GFP-tagged CD63 under control of the UAS promoter (CORRIGAN *et al.* 2014)..CD63 is a vertebrate tetraspanin protein that is present at a very high concentration inside the exosomes. Inside the cells CD63 localizes to the multivesicular bodies (MVBs) and lysosomes and smaller amounts are present in the plasma membrane (MITTELBRUNN *et al.* 2011). Unfortunately, there is a lack of consensus on which Drosophila proteins can be used as exosomal markers. *UAS-CD63-GFP* flies were crossed with *cg-gal4* flies that express *gal4* in the fat body. Fat body tissue from the progeny (wandering third instar larvae) of the cross was isolated and immunostained with anti-GFP antiserum to verify that the multivesicular body and lysosomes in the fat body of these animals have CD63-GFP incorporated in them. Our results demonstrate that only fat body from experimental animals has GFP-labeled membranous structures, whereas fat body from the control animals lack staining (Figure 16).

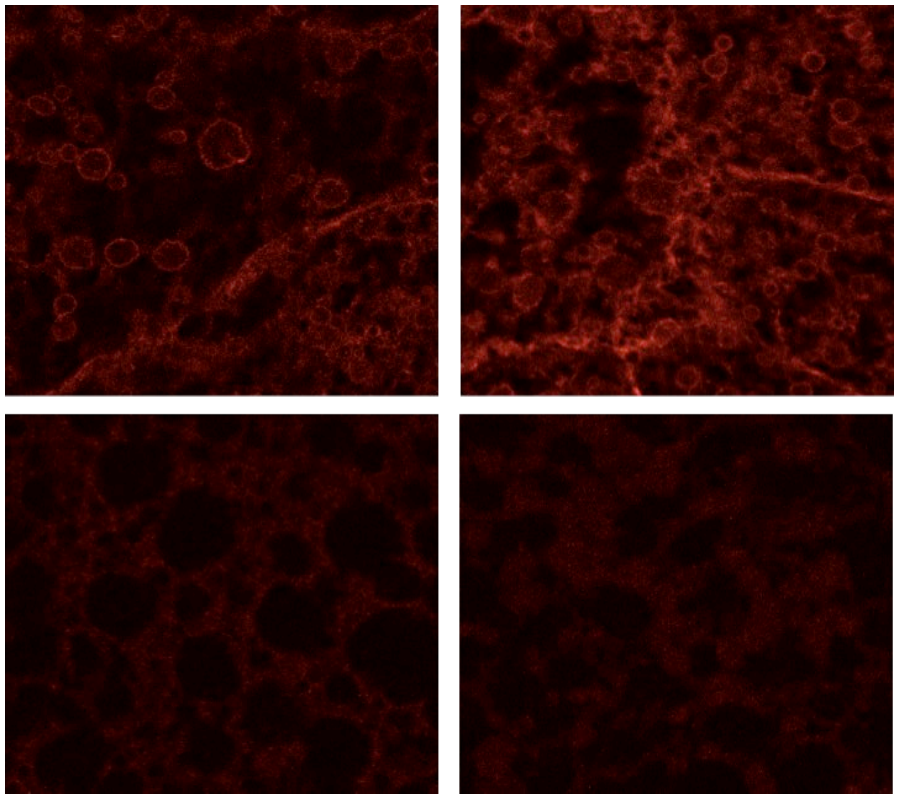

Figure 16: Fat body immunostained with anti-GFP antisera. A) Fat body from two independent larvae of the genotype *cg-gal4>UAS-CD63-GFP* showing GFP labelled membranous structures. **B)** Fat body from *UAS-CD63-GFP* reporter-only flies. Images taken at 100x using a confocal microscope.

4.4 Discussion

Past studies have demonstrated that diffusible factors released by tissues other than the nervous system contribute to standard courtship behavior (SIWICKI *et al.* 2005; LAZAREVA *et al.* 2007). Since fat body-secreted factors can have non-cell autonomous effects that, in turn, impact behavioral responses (LAZAREVA *et al.* 2007), we hypothesized that fat body secreted miRNAs in the hemolymph play a role in optimizing courtship behavior. We utilized a *dcr-1* knockdown approach to show that fat body miRNAs are important for courtship regulation in *Drosophila melanogaster* males. Reduction of *dcr-1* in the fat body resulted in lower CI in Drosophila males. A similar *dcr-1* knockdown approach was adopted by Barrio et al. (2014) to test the effect of reducing miRNA biogenesis in the fat body on starvation resistance in adult flies. They observed that adult flies expressing *dcr-1* RNAi in the fat body had higher survival rates under conditions of nutrient deprivation.

Besides evaluating the systemic impact of reducing miRNA biogenesis in the fat body on courtship activity of males, we wanted to further understand whether secretion of fat body miRNAs contributes to courtship behavior. To this end, first we demonstrated that miRNAs are indeed present in circulation and that fat body can secrete miRNAs.

To investigate non-cell autonomous impacts of fat body miRNAs, we are attempting to stop regulated release of miRNAs from the fat body by blocking exosome secretion. Inhibiting exocytosis should achieve the desired effect since exosomes, a major carrier for miRNAs in biofluids, are released via exocytosis. Corrigan et al. (2014)

showed that reducing two components of the Exosomal Sorting Complex Required for Transport (ESCRT), ALiX and Rab11, significantly reduced the number of exosomes released by secondary cells in Drosophila accessory glands. Currently, I am in the process of calculating CI for animals with components of the ESCRT (AliX and Rab11) reduced in the fat body. *Cg-Gal4>UAS-ALiX-RNAi* flies and the dominant negative allele for Rab11 (DNRab11) have been used to reduce expression of these components of the ESCRT complex in fat body.

In order to understand vesicle-mediated exchange between fat body and other tissues we attempted to track GFP-tagged exosomes and determine whether these could be taken up by recipient tissues. One of the problems we faced with this approach is that most of the known fat body drivers have a high level of expression in the CNS too. We tested a variety of fat body drivers-- *cg-gal4, r4-gal4, ppl-gal4* and *lsp-gal4*--but none of these drivers had expression restricted to the fat body, making it difficult to use these drivers to track uptake of fat body-expressed exosomes by the CNS.

Although tracking fat body secreted exosomes in vivo has proven to be challenging, our ex vivo fat body culture experiments combined with exosomal staining experiments provide some indication that in-vitro approaches such as organ co-culture experiments can be used to identify exchange of exosomes between different tissues. It must be noted that even if exosome tracking tools are developed, it will be challenging to show that any effects of exosome uptake can be attributed to the miRNA cargo. Understanding the impact of miRNAs delivered via exosomes will require that the

recipient cells lack the miRNA transported by the vesicles and demonstrating that the effects are strictly due to the miRNA contents of the vesicle.

5. SUMMARY AND CONCLUSIONS

When we started this study little was known about the role of miRNAs in the regulation of behavior. Li et al. (2013) had shown that miRNAs can affect memory formation and a few studies had implicated the role of miRNAs in circadian regulation. In the past 5 years there has been a dramatic increase in the number of studies done to explore the contribution of miRNAs to biological processes beyond development, especially in *Drosophila melanogaster*. Our study, however, will be the first to our knowledge to carry out a genome-wide screen to demonstrate the role of miRNAs in the regulation of a complex social behavior like courtship.

We carried out a large-scale genetic screen with miRNA knockout mutant males to identify candidates that cause aberrations in various parameters of male courtship. We selected this behavior because it is robust, and the underlying sex-determination genetic hierarchy is well characterized. Courtship and mating behavior is innate and stereotypical, yet it is modified in response to environmental cues. The screen therefore provided us an excellent platform to study miRNA contribution to a multifaceted phenotype that requires integration of genetic and environmental cues. The parameters scored for the screen were impacted by both the effectiveness of male courtship attempts and the courtship subjects' response. In the initial screen ~26% of the strains tested had reduced mating success, while ~39% showed variations in other parameters. We further analyzed two of the candidates (*miR-263b* and *miR-317*). *miR-263b* mutants courted females significantly more than wildtype males, and the higher CI phenotype was

maintained in the deficiency transheterozygotes and miRNA sponge-expressing mutants. Additionally, *miR-317* KO mutants, deficiency transheterozygotes and miRNA sponge animals had lower mating success than wildtype males. Through this study we have been able to evaluate the effect of complete genetic ablation of a class of post-transcriptional regulatory elements that is relatively under explored in the context of behavior and observed the impact of miRNA removal on aspects of courtship behavior that are influenced by both a male's courtship rigor as well as his attractiveness.

Besides testing the standard male-female courtship behavior parameters, we also tested for male-male courtship in grouped males, as that is another well-studied phenotype observed due to disruptions in the sex-determination circuitry. Remarkably, *miR-957* mutants displayed high levels of male-male courtship. We showed that these males vigorously courted males but did not elicit courtship from other males. This indicated that the pheromonal profile of *miR-957* KO males had not changed but, instead, that they failed to perceive sexual cues from courtship subjects correctly. Further experiments conducted to understand this behavior indicated that the perception of female attractive pheromones was intact, but *miR-957* mutants failed to recognize male pheromones as aversive. To determine if *miR-957* KO males are not sensing male aversive cues correctly, we tested their courtship towards female subjects which are known to have high levels of male courtship inhibitory pheromones. By performing behavioral assays and perfuming experiments we found that the absence of the *miR-957* led to compromised sensing and evaluation of the aversive pheromones such as 7-T and cVA. These results demonstrated that a single miRNA can contribute to the processing

of sensory information that is important for maintenance of sex and species-specific courtship barriers and chemical mate guarding. This finding is in line with the fact that *miR-957* is conserved across Drosophila species, because behavioral reproductive isolation operates across the species as it is rare to find interspecies hybirds in nature (BARBASH 2010). Studies have suggested that the reproductive isolation is not due solely to reduced survival of hybrids but also that neurobiological mechanisms that prevent interspecific courtship are important contributors to maintaining such barriers (DUKAS 2004).

We further reduced *miR-957* expression in specific neuronal populations and tested for the male-male courtship phenotype. We found *miR-957* expression to be important in the mushroom body as well as in *doublesex*-expressing neurons. Neurons that express members of the Drosophila sex-determination hierarchy, such as *doublesex,* primarily control Drosophila courtship behavior. This circuit is responsive to sensory stimuli making courtship behaviors modifiable in response to attractive or aversive stimuli.

In Drosophila, behavioral regulation involves communication between different organs via secreted factors. There is evidence for miRNA exchange between cells and tissues, and we have shown that miRNAs can be detected in Drosophila hemolymph. We have also provided data that suggests the fat body secretes miRNAs. In order to understand the relevance of these secreted miRNAs we have laid the ground work for developing tools that can be used to track exosome-mediated communication between tissues.

We wondered if apart from the tissue autonomous effects of miRNA reduction, another consequence is a hindrance in communication between multiple tissues. Therefore, in addition to evaluating the behavioral outcomes of reduction in miRNA expression in whole animals, we are also trying to understand if fat body expressed miRNAs are important for Drosophila male courtship behavior. We showed that reducing miRNA biogenesis in the fat body lowers courtship efforts by males and are currently evaluating if reducing secretion of these miRNAs has a similar effect on courtship.

To summarize, previous studies explored the roles of proteins in development and maintenance of sex-specific characteristics in Drosophila, but less is known about additional layers of regulation in this context, such as those imposed by non-coding RNAs. Our results have paved the way to increase our understanding of how critical mechanisms of communication in insects, such as pheromone sensing, are regulated by multiple layers of post-transcriptional control. My study has provided new insights into the possible roles of miRNAs in regulation of complex physiological and behavioral processes in adult *D. melanogaster*.

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