*fit*TING IT ALL TOGETHER: HOW COURTSHIP- AND MATING-RESPONSIVE GENES AFFECT *Drosophila melanogaster* MALE BEHAVIOR

A Dissertation

by

LISA LYNN ELLIS

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 2010

Major Subject: Biology

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Approved by:

Chair of Committee,	Ginger E. Carney
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ABSTRACT

*fit*ting It All Together: How Courtship- And Mating-Responsive Genes Affect *Drosophila melanogaster* Male Behavior. (August 2010)

Lisa Lynn Ellis, B.S. with Honors, Sam Houston State University

Chair of Advisory Committee: Dr. Ginger E. Carney

Behavior is a complex process resulting from the integration of genetic and environmental information. Thus, the genetically tractable *Drosophila melanogaster* was utilized to better understand the interplay between these factors since Drosophila males and females exhibit sex-specific courtship behaviors that are innate yet modifiable. These sex-specific behaviors, as well as sexually dimorphic development, are regulated, in part, by the somatic sex-determination hierarchy.

Since reproductive behaviors rely on the rapid integration of multiple sensory cues, it is likely that the perception and integration of such cues and mating-induced physiological changes are mediated in part by changes in gene expression. Therefore, it was hypothesized that assaying gene expression changes in response to courtship or mating in Drosophila males would uncover new targets of the sex-determination hierarchy and other behaviorally important loci. We took a novel approach to find these behaviorally-responsive loci by utilizing microarray technology to assess courtship- or mating-induced gene expression changes in Drosophila male whole bodies or heads.

Mutations in candidate loci were tested for effects on reproductive behaviors and

present the first data showing that *egghead* (*egh*) and *female-specific independent of transformer* (*fit*) affect male reproductive behavior. *egh* is up regulated in male heads 20 min after courting and is required post-developmentally in a subset of neurons for robust male courtship behavior. *fit*, a fat body-expressed sex-determination hierarchy target gene, is up regulated in male whole bodies after 5 min of courtship. *fit* is also up regulated in male heads after 20 min of courtship or 2 hrs after mating. Mutations in *fit* result in male-male courtship; more specifically, *fit* mutants direct courtship towards males and also elicit courtship from wild-type males. By analyzing *fit's* role in courtship behavior, we also shed light on the role the fat body plays in modulating behavior.

These studies provide the first pieces of evidence that gene expression changes occur in Drosophila males performing reproductive behaviors. This novel approach identified behaviorally important loci that are expressed in the nervous system and the fat body, indicating that both tissues modulate behavior. Also identified were sex-determination hierarchy target genes and it is likely that further analysis of the remaining candidates will reveal more members of this genetic cascade.

DEDICATION

This work is dedicated to all the wonderful teachers of academics and life lessons I've encountered in my journey. May your desire to teach and your enthusiasm for learning be contagious. I'm blessed to have known you and to follow in your footsteps.

ACKNOWLEDGEMENTS

This dissertation work would not have been possible without the support of many people. First and foremost, I would like to acknowledge and thank my advisor, Dr. Ginger E. Carney. She has been an exceptional role model. Dr. Carney has fostered my growth and independence as a scientist and offered her patient guidance along the way. Without her unwavering support and eternal encouragement I would not have made such contributions to the scientific community, but most importantly I would not have enjoyed my graduate experience as much as I have.

I'd also like to thank my committee members, Drs. Bruce B. Riley, Arne C. Lekven and Vladislav Panin. Their comments and questions helped make me a better scientist and have broadened my perspective.

I would like to thank all of my labmates, past and present, for being such great friends and for making the lab an enjoyable place to work. Kara Boltz has helped me tremendously. When I started in the Carney Lab, she was a great mentor. Kara also contributed to my research by working on the *fit* cloning projects. I have been fortunate to mentor and work with two junior graduate students, Christoph Schwedes and Sehresh Saleem. Both bring an air of creativity and fresh ideas to the lab. I wish them all the success they so greatly deserve. I was also fortunate to mentor many undergraduates during my time in the Carney Lab. They have been great friends and terrific sounding boards. Working tirelessly on their own projects, they found time to contribute to mine. In particular, I'd like to thank Rosemary Neyin for collecting the *Jhe* behavioral data.

Meredith Pinto and Stephanie Grady, who are skilled fly dissectors, helped me on many occasions with immunohistochemistry experiments.

I would also like to acknowledge the camaraderie within the Department of Biology. It has been a pleasure to meet and learn from such wonderful faculty, staff, and students. To those graduate students senior to me, I want to thank you for your helpful hints and the examples you set. I hope that I have done such a wonderful job for the remaining graduate students.

Outside of Texas A&M, I have had a great support staff of friends and family throughout my lifetime. They have kept me grounded and served as an outlet, helping me focus my thoughts elsewhere when such an occasion was warranted. I have had the privilege of learning from many great teachers and professors; however, there are two that I owe a special thanks to. I would not be a biologist without the great dedication and extraordinary teaching skills of Denise Haynes. Even when I thought I'd be a computer scientist, Denise know I'd end up a biologist. Kelly Jones has been a great role model and a wonderful source of encouragement and continual support all these years.

Finally, the utmost thanks belong to my family. The love and support they have shown me my entire life is immeasurable. I am indebted to them for my confidence and for my humility. My mom and dad instilled in me a desire to learn, but also the reality that I do not always know the answer. I certainly wouldn’t have made it this far without them. I love them more than words can say and can never repay them for all they have done.

NOMENCLATURE

ap	*apterous*, subset of central nervous system neurons
CI	Courtship Index; time in courtship/observation time
clt	*cricklet*
CNS	Central nervous system
CS	*Canton-S*, a wild-type *D. melanogaster* strain
egh	*egghead*
fit	*female-specific independent of transformer*
Gal4	yeast transcription factor that binds to UAS sites
GCOS	GeneChip® Operating System, microarray extraction algorithm
GCRMA	Guanine Cytosine Robust Multi-Array Analysis, microarray extraction algorithm
Jhe	Juvenile hormone esterase
PM	Perfect-Match, microarray extraction algorithm
PM-MM	Perfect-Match Mismatch, microarray extraction algorithm
qPCR	quantitative polymerase chain reaction
SDH	sex-determination hierarchy
Sim2	A wild-caught *D. simulans* strain
UAS	upstream activating sequence, binding site for Gal4

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CHAPTER I

INTRODUCTION

Understanding the genetic regulation of behavior

The proper integration, processing and transmission of sensory information is vital for an appropriate behavioral response. Since genetic and environmental components affect these processes, we were interested in assaying courtship- or mating-induced gene expression changes in *Drosophila melanogaster* males. By studying these tissue-specific behaviorally-responsive loci, we can better understand the genetic regulation of behavior.

Behavior and gene expression can be modified by social interactions

As we work to understand how behaviors are regulated, we must consider both the genetic and environmental influences on behavior. In addition to how behavioral output is affected, we should also consider how the performance of a behavior affects the organism, including affecting subsequent behaviors and modifying gene expression.

The social context of an organism's environment is known to affect behavior and gene expression and examples of this phenomenon are found across taxa. For example, tadpoles in the presence of predators increase expression of body morphology-related genes that are linked to the tadpoles' morphological change to the bulgy phenotype (Mori *et al.* 2005). Similarly, as cichlid males usurp the dominant role within a

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This dissertation follows the style of *Genetics*.

population *early growth response* (*egr-1*) is rapidly (within 20 min) up regulated and males transition to the dominant color pattern and behavior (Burmeister *et al.* 2005).

Courtship behavior is also affected by social cues. Zebra finch female mate choice, also known as sexual partner preference, is influenced by estrogen levels and group dynamics (male to female ratio and tactile contact) (Adkins-Regan 2005). Also, differential expression of the *egr-1* homolog, ZENK, correlates with group dynamics (presence of females, males or isolation) and courtship behavior (singing and dancing towards females or singing when around males or alone) (Jarvis *et al.* 1998).

As with other organisms, Drosophila behaviors and neural connections are influenced by social interactions. A fly's circadian rhythm changed depending on which flies he was interacting with. Wild-type flies housed together showed robust synchronization but this rhythm was disrupted when *clock* mutants were added to the group (Levine *et al.* 2002). Group dynamics also affected aggressive behavior of both sexes. Females raised in isolation showed increased aggressiveness compared to females housed in groups (Ueda and Kidokoro 2002). The number of aggressive bouts and success in those bouts altered male fighting repertoires (Yurkovic *et al.* 2006). As larval population density increased, the number of boutons at the neuromuscular junction decreased (Stewart and McLean 2004).

Social interactions also affect Drosophila courtship behaviors. As males interacted with other males, they learned to avoid courting males (Gailey *et al.* 1982; Mehren and Griffith 2004); older males show decreased male-male courtship (Svetec *et al.* 2005; Svetec and Ferveur 2005). Males also decreased courtship towards females

after courting unreceptive females (Siegel and Hall 1979; Gailey *et al.* 1984; Joiner and Griffith 1997; Joiner and Griffith 1999; Kamyshev *et al.* 1999; Joiner and Griffith 2000; Mehren and Griffith 2004).

Although we know social environment affects behavior, little is known about how it impacts fly gene expression patterns. Our microarray and subsequent behavioral analyses provide the first evidence that courtship alone or courtship followed by mating affects male gene expression and that responsive loci impact behavior.

Drosophila melanogaster as a tool for understanding behavior

We can utilize the model organism *Drosophila melanogaster*, which has proven to be a valuable research tool, to better understand the genetic regulation of behavior. Several Nobel laureates have utilized this model organism to study genetics (Thomas H. Morgan, 1933), mutagenesis (Hermann Muller, 1946), development (Edward B. Lewis, Christiane Nüsslein-Volhard and Eric Wieschaus, 1995), or olfaction (Richard Axel and Linda Buck, 2004).

In addition to the plethora of research questions one can address using Drosophila, fruit fly husbandry is a relatively simple process compared to that of mice and other multi-cellular organisms. The generation time from embryo to adult is 10 days at 25^{o}C, and one mating can produce hundreds of offspring. In addition to the ease of caretaking, fruit flies are a powerful genetic tool. With a sequenced and annotated genome, forward and reverse genetic techniques are possible. One can conduct the typical forward genetic screens by exposing flies to the ethanemethylsulfonate (EMS)

mutagen or utilizing P-element transposition. There are many reverse genetic approaches available to fly geneticists, including analyzing P-element insertions, RNA interference (RNAi), and homologous recombination.

The Gal4-UAS system is also utilized by fly geneticists. This yeast paradigm allows targeted expression of gene constructs for reduced or increased expression (Brand and Perrimon 1993). Expression of the Gal4 transcription factor is under control of a promoter sequence. This construct is usually referred to as the "driver". Depending on the promoter sequence, one can control the timing or cell-specific activation of Gal4. The transcription factor recognizes and binds to the Upstream Activating Sequence (UAS), which facilitates transcription of the gene sequence fused downstream of the UAS (Figure 1). Often this construct is referred to as the "reporter" or the "responder" depending on what is being expressed under control of the UAS. The temperature sensitive Gal80 ($Gal80^{ts}$) allele can be added to the system to further control when Gal4 is activated and when the responder is activated. At the permissive temperature of $20^{o}C$, Gal4 is bound to the UAS, but $Gal80^{ts}$ is bound to Gal4, preventing transcription. Thus the responder is not expressed. However, shifting to the restrictive temperature of $29^{o}C$ dissociates $Gal80^{ts}$ from Gal4, activating the responder (McGuire *et al.* 2004).

Reproductive behaviors of Drosophila males

Drosophila melanogaster exhibit complex sex-specific stereotypical reproductive behaviors that are mediated by multiple sensory modalities. Mating success relies on the perception and interpretation of olfactory, gustatory, auditory, tactile and

visual cues. Courtship is initiated when the male orients toward the female. He will then follow the female as she runs away. As the male taps the female's back legs with his

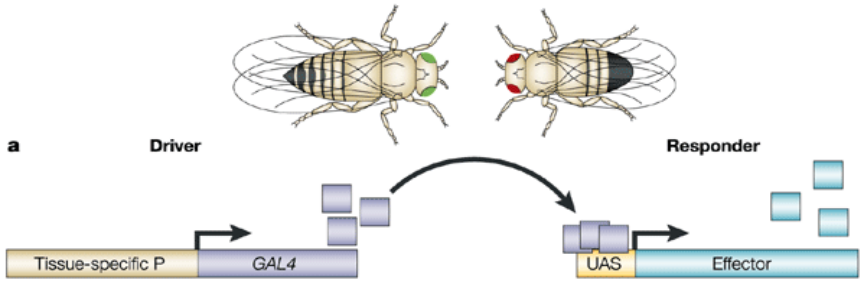

Figure 1. The binary Gal4/UAS system. Modified from Wimmer 2003.

forelegs he receives chemosensory cues regarding the female's mating status. Virgin females exhibit different pheromonal profiles compared to recently mated females, and newly eclosed females reject courtship advances the first day after eclosion (Manning 1966; Manning 1967). The male vibrates his wings to produce a species-specific song for the female and she in turn receives auditory cues to determine if he is a suitable mate. Chemosensory information is received as the male licks the female's genitals and curls his abdomen for attempted copulation. If the female finds the male to be a suitable mate, she will open her vaginal plates and allow the male to copulate with her (Hall 1994; Greenspan 1995) (Figure 2).

During mating, the male transfers Sex peptide and other accessory gland proteins in his ejaculate to the female. These proteins cause behavioral and physiological changes in the female to benefit the male's reproductive fitness. Mated females increase ovulation and egg laying and are unattractive to males (Chen *et al.* 1988; Aigaki *et al.*

1991; Kubli 1992; Wolfner 1997; Tram and Wolfner 1998; Heifetz *et al.* 2000; Fleischmann *et al.* 2001; Chapman *et al.* 2003; Liu and Kubli 2003; Heifetz *et al.* 2005; Peng *et al.* 2005; Soller *et al.* 2006).

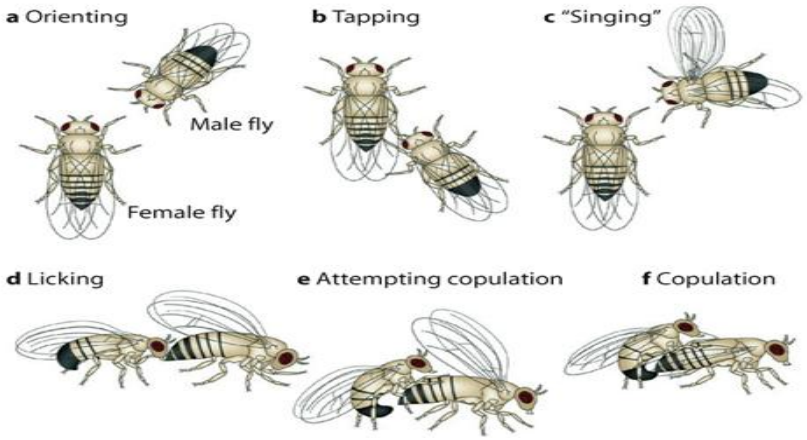

Figure 2. *Drosophila melanogaster* courtship ritual. Sokolowski 2001

The Drosophila somatic sex-determination hierarchy and its regulation of morphology and behavior

The somatic sex-determination hierarchy (SDH) is the major genetic pathway regulating sexually dimorphic development and behavior (Figure 3) (reviewed in Cline and Meyer 1996). Atop this hierarchy is the splicing factor Sex-lethal (Sxl) which only functions in females (Cline 1984). Therefore, in females Sxl binds to *transformer (tra)* pre-mRNA and blocks the 3' non-specific splice site resulting in production of functional Tra. Males lack functional Sex-lethal and Tra (Boggs *et al.* 1987; McKeown *et al.* 1987; Bell *et al.* 1988; McKeown *et al.* 1988; Nagoshi *et al.* 1988; Sosnowski *et al.* 1989). Tra is also a splicing factor that works in conjuction with the sex non-specific constitutively expressed Transformer-2 (Tra-2) (Goralski *et al.* 1989; Hedley and Maniatis 1991; Tian

and Maniatis 1993; Amrein *et al.* 1994; Lynch and Maniatis 1995). In females, Tra/Tra-2 splicing activity leads to a truncated *fruitless (fru)* transcript (and presumably non-functional Fru protein), and the female-specific *doublesex (dsx)* transcript which leads to the female-specific DsxF protein. Since males lack Tra, the default splicing of *fru* and *dsx* result in the male-specific isoforms FruM and DsxM, respectively (Burtis and Baker 1989; Ito *et al.* 1996; Heinrichs *et al.* 1998). DsxF, DsxM, and FruM are transcription factors (Burtis *et al.* 1991; Erdman and Burtis 1993; Ito *et al.* 1996; Ryner *et al.* 1996). We know some of their downstream targets (Arbeitman *et al.* 2004; Dalton *et al.* 2009; Goldman and Arbeitman 2007; Lebo *et al.* 2009) though the functions of these targets are poorly characterized.

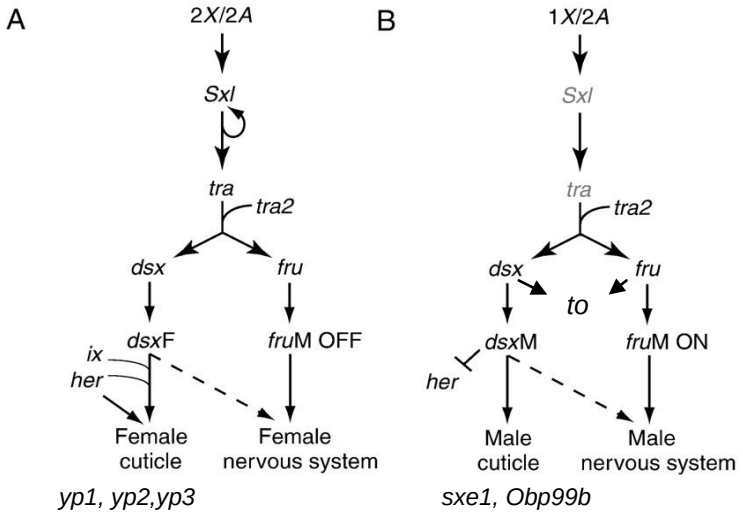

Figure 3. The Drosophila somatic sex-determination hierarchy regulates somatic tissue development and courtship behavior. Sxl is the upstream regulator of this pathway; it is expressed in females (A) and absent in males (B). Males lack Tra and thus default splicing of *fru* and *dsx* occurs, yielding male-specific isoforms of Fru and Dsx. Modified from Shirangi and McKeown 2007.

One of the best characterized SDH genes is *fru*. FruM is necessary and sufficient

for male courtship behavior and directs development of the sexually dimorphic central and peripheral nervous systems (Demir and Dickson 2005; Kimura *et al.* 2005; Stockinger *et al.* 2005; Billeter *et al.* 2006; Mellert *et al.* 2010). Decreased expression of *fruM* results in female-like nervous systems (Kimura *et al.* 2005) and multiple courtship-related phenotypes including loss of courtship towards females (Ito *et al.* 1996; Ryner *et al.* 1996; Villella *et al.* 1997), reduced male-female courtship (Ryner *et al.* 1996; Villella *et al.* 1997; Demir and Dickson 2005; Stockinger *et al.* 2005; Manoli *et al.* 2005; Billeter *et al.* 2006), increased male-male courtship (Gailey and Hall 1989; Ryner *et al.* 1996; Villella *et al.* 1997; Demir and Dickson 2005; Stockinger *et al.* 2005; Billeter *et al.* 2006), and courtship chaining (Villella *et al.* 1997; Demir and Dickson 2005; Stockinger *et al.* 2005; Billeter *et al.* 2006). Mutations in *fru* also affect wing extensions, (Wheeler *et al.* 1989; Villella *et al.* 1997; Billeter *et al.* 2006; Koganezawa *et al.* 2010), male-male habituation (Manoli *et al.* 2005), courtship conditioning (Manoli *et al.* 2005), and partner preference. *fru* mutant partner preference can be female- or male-biased, and some mutant males court each sex equally (Villella *et al.* 1997). Certain *fru* mutant males have changes in pheromone profiles resulting in male-male courtship. As expected, females expressing FruM exhibit male-like courtship and rejection behaviors (Demir and Dickson 2005; Manoli *et al.* 2005). In addition to affecting courtship behaviors, *fru* mutant males exhibit increased aggression (Lee and Hall 2000; Vrontou *et al.* 2006).

Not all members of the SDH are sex-specifically expressed. *tra*, but not *fru*, regulates *dissatisfaction* (*dsf*) expression. *dsf* is expressed and functional in both sexes

and mutations in *dsf* affect male and female behaviors. Mutant *dsf* males are bisexual and form courtship chains similarly to *fru* mutants. Males also have problems bending their abdomens during courtship resulting in increased mating latencies. In females, *dsf* is needed for female receptivity and oviposition (Finley *et al.* 1997; Finley *et al.* 1998; Yamamoto *et al.* 1998).

SDH target genes with characterized function include *yolk proteins* (*yp1, yp2,*and *yp3*), *sex-enzyme 1* (*sxe1*) and *Odorant binding protein 99b* (*Obp99b*). *yp1, yp2,* and *yp3* are involved in egg development and are regulated by DsxF and Hermaphrodite (Her). DsxM blocks *yolk protein* expression in males (Bownes *et al.* 1983; Belote *et al.* 1985; Burtis *et al.* 1991; Li and Baker 1998; Garrett-Engele *et al.* 2002). DsxM activates *sxe1,* a fatty-acid ω-hydroxylase needed for male courtship and mating (Fujii and Amrein 2002; Fujii *et al.* 2008). *Obp99b* is also regulated by DsxM. Overexpressing *Obp99b* in females results in decreased mating success most likely because these transgenic females run away from the males (Fujii and Amrein 2002).

The Dsx and Fru branches of the SDH converge to regulate *takeout* (*to*) and *retained* (*retn*). DsxM and FruM promote *to* expression in males while DsxF represses *to*. There is a greater reduction of male courtship in to^{-}*;fru/+* males compared to to^{-} males (Dauwalder *et al.* 2002). *retn* regulates neural development (Shandala *et al.* 2003; Ditch *et al.* 2005) and interacts with *fru* and *dsx* to modulate male and female behavior. *retn* suppresses male courtship by antagonizing *fruM*. Females with reduced *retn* and *dsxF* have increased mating latencies and exhibit bisexual courtship (Shirangi *et al.* 2006).

Similarly to *dsf*, the SDH target gene *female-specific independent of transformer (fit*) is expressed in both sexes. Though the name implies that *fit* is expressed only in females, this is not the case. *fit* is expressed in males but may be expressed at higher levels in females. Another ambiguity regarding *fit* is its placement within the SDH. *Sxl* seems to regulate *fit,* though as the name implies, *tra* does not (Fujii and Amrein 2002). However, there is conflicting evidence showing that *fit* is dependent on *tra* expression (Evans and Cline 2005; Goldman and Arbeitman 2007).

The Drosophila nervous system

Behavior requires the proper perception and integration of environmental cues. With regard to courtship, multiple sensory cues need to be processed for proper courtship. The perception of these cues occurs mostly in the peripheral nervous system (PNS) and the information is processed in the central nervous system (CNS).

The Drosophila CNS is comprised of the brain and ventral nerve cord. The brain is segregated into the optic lobe, the subesophageal ganglion (SOG), and the central brain. The optic lobe receives and processes visual stimuli while gustatory cues are interpreted by SOG neurons. Within the central brain, the antennal lobes receive olfactory neuron projections from the antennae and maxillary palps (Amrein 2004). The mushroom bodies, also part of the central brain, are involved in integrating the information, and their function is imperative for memory and learning (reviewed in Davis 1993). Signaling within the pedunculus of the mushroom body has been implicated in functioning in female-directed courtship behavior (Sakai and Kitamoto

2006). Within the central brain, the central complex is made up of the protocerebral bridge, fan-shaped body and ellipsoid body. Fan-shaped body neurons seem to regulate male courtship behavior regardless of the courtship object (Sakai and Kitamoto 2006). Disruption of central complex neurons result in abnormal courtship songs and thus decreased courtship levels (Popov *et al.* 2003; Popov *et al.* 2005).

Non-neuronal tissue modulates behavior

The nervous system is not the only tissue that governs reproductive behavior. The Drosophila adipose tissue known as the fat body is a secretory tissue that modulates metabolic processes (reviewed in Schlegel and Stainier 2007) and has recently been implicated in modulating behavior (Dauwalder *et al.* 2002; Fujii and Amrein 2002; Lazareva *et al.* 2007; Fujii *et al.* 2008; Benito *et al.* 2010; reviewed in Dauwalder 2008). The SDH target genes *sxe1* and *to* are expressed in the fat body; however, the mechanisms by which these genes regulate courtship behavior are unclear. It is likely that signaling from the fat body (either directly or by secretion into the hemolymph) affects nervous system or genital tract function to modulate behavior.

Oenocytes are also non-neuronal cells implicated in modulating behavior, including sex and species recognition. These cells line the abdominal cuticle and are the presumptive site of hydrocarbon biosynthesis. Some hydrocarbons act as sex pheromones, and the numbers of carbon atoms and double bonds and the position of these double bonds encode sex- and species-specific information (Jallon 1984; Coyne *et al.* 1994; Ferveur 1997; Ferveur *et al.* 1997; Savarit *et al.* 1999; Ferveur 2005). For

example, *Drosophila melanogaster* profiles differ from those of other Drosophilid species. *D. melanogaster* males generally express higher levels of monoalkenes, notably 7-tricosene (7-T); 7-T inhibits courtship from males and promotes female receptivity of male mating advances (Grillet *et al.* 2006; Lacaille *et al.* 2007). *D. melanogaster* females predominantly express dialkenes, notably 7,11-heptacosadiene (7,11-HD) and 7,11-nonacosadiene (7,11-ND); 7,11-HD and 7,11-ND stimulate courtship from males (Antony *et al.* 1985).

The synthesis and maturation of these hydrocarbons are carried out by enzymes such as *desaturase 1 (desat1)*. It is unclear whether or not expression of *desat1* and other hydrocarbon biosynthetic enzymes is required in oenocytes to regulate pheromone production. Decreased expression of *desat1* results in pheromone profile changes but this reduced expression is not restricted to oenocytes (Dellerac *et al.* 2000; Marcillac *et al.* 2005; Ueyama *et al.* 2005; Chertemps *et al.* 2006; Krupp *et al.* 2008). The ablation of oenocytes usually includes the disruption of fat body signaling and thus it has been difficult to separate fat body and oenocyte functions in pheromone synthesis (Billeter *et al.* 2009).

Dissertation objectives

The objective of this dissertation is to elucidate the genetic underpinnings of male Drosophila behavior by identifying courtship- and mating-induced gene expression changes and characterizing the roles of candidate genes in male courtship behavior.

Chapter II addresses the species-specific changes occurring in male whole bodies after 5 min of courtship. Chapter III focuses on gene expression changes in the heads of males after 20 min of courtship. I also show that the courtship-responsive gene *egghead* (*egh*) is required post-developmentally in a subset of neurons for male courtship behavior. The role of the fat body-expressed courtship- and mating-responsive gene *female-specific independent of transformer* (*fit*) in repressing male-male courtship behavior is detailed in Chapter IV. In Chapter V, I discuss the post-mating gene expression changes that occur in male Drosophila heads and the functions of *Juvenile hormone esterase* (*Jhe*) in male reproductive behavior. A discussion of the data and future implications are dealt with in Chapter VI.

CHAPTER II

Drosophila melanogaster MALES RESPOND DIFFERENTLY AT THE BEHAVIORAL AND GENOME-WIDE LEVELS TO *Drosophila melanogaster* AND *Drosophila simulans* FEMALES*

Introduction

In order to pass their genetic material to the next generation, it is imperative to organisms that they choose an appropriate individual with which to mate. Sometimes choosing a mate is not a simple task, particularly when different species with overlapping ecological distributions have similar phenotypic characteristics, possibly making it difficult to discern different species. Animals have developed sensory systems that allow them to distinguish conspecific individuals and heterospecifics that are morphologically similar. For instance, humans rely primarily on visual signals to distinguish individuals, whereas mice rely more heavily on olfactory cues to choose mates. Members of the genus Drosophila, which use a variety of sensory systems to select mates, have served as an excellent model for defining the molecules and neural mechanisms that are used to make such determinations.

Drosophila males perform elegant, species-specific courtship rituals in order to entice conspecific females to mate and reproduce. The courtship of *Drosophila*

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*Reprinted with permission from "*Drosophila melanogaster* males respond differently at the behavioral and genome-wide levels to *Drosophila melanogaster* and *Drosophila simulans* females" by L. L. Ellis and G. E. Carney, 2009. *Journal of Evolutionary Biology*, 22, 2183-2191, Copyright 2009 by Wiley-Blackwell.

melanogaster is the best characterized of the Drosophila species in terms of the behaviours performed as well as the genetic mechanisms that underlie sex-specific behaviours. *Drosophila melanogaster* use all of their sensory modalities (visual, olfactory, gustatory, auditory and tactile) during courtship. In laboratory courtship assays, the male performs a stereotypical suite of behaviours towards his female that begins with identification and orientation towards the female courtship object. He follows his chosen female, taps her abdomen with his front legs, extends and vibrates a wing to produce a species-specific courtship 'song', licks the female's genitalia, bends his abdomen, and mounts the female for copulation (reviewed by Greenspan and Ferveur 2000).

Sexually mature Drosophila are genetically programmed to perform reproductive behaviours, but these behaviours are modifiable by experience. Flies raised in isolation until adulthood perform the standard reproductive behaviours, indicating that genes play an important role in behavioural specification. However, it is clear for males that social experience with both male and female courtship objects affects innate behaviour (reviewed by Ewing 1983; Tompkins 1984; Greenspan and Ferveur 2000; Siwicki and Ladewski 2003; Mehren *et al.* 2004). Inexperienced males initially show robust courtship towards immature males, but this response decreases over time (Gailey *et al.* 1982). A male that encounters an unreceptive female decreases later courtship towards any female, even those that are receptive (Siegel and Hall 1979; Gailey *et al.* 1982; Kamyshev *et al.* 1999), and *D. melanogaster* males have the ability to learn to avoid heterospecific females (Dukas 2004).

Although female behaviours are subtler, females are not passive participants in the courtship process. Initially, females run away from courting males, but if they are receptive they slow down, lift their abdomens, open their vaginal plates and allow the male to copulate (reviewed by Greenspan and Ferveur 2000).

In the wild, *D. melanogaster* coexist with the closely related species *Drosophila simulans*. This overlap in species distributions can in principle lead to heterospecific mating opportunities. To the human eye, females of the two species are virtually indistinguishable, whereas males differ in the shape of their genitalia. However, other differences exist between these two species that may reduce the probability of heterospecific matings. For instance, there are species-specific and, in the case of *D. melanogaster*, sex-specific differences in the predominant cuticular hydrocarbon (Jallon 1984; Jallon and David 1987). *Drosophila melanogaster* cuticular hydrocarbon profiles are used by males of this species to distinguish mature males, receptive females and mated females from one another (Scott 1986; Vaias *et al.* 1993; Siwicki *et al.* 2005).

Hydrocarbon cues may be less important for female choice, which appears to be partially dictated by the male's song (Tomaru *et al.* 2000). Species differences exist in the two elements comprising the courtship song: the sine song and the interpulse interval (reviewed by Greenspan and Ferveur 2000; Tauber and Eberl 2003). Females' ability to differentiate conspecific and heterospecific courtship songs can lead to sexual isolation for sympatric Drosophila species (Doi *et al.* 2001; Yamada *et al.* 2002). Females generally prefer the courtship songs of conspecific males (Ritchie *et al.* 1999), although there is overlap in courtship song frequencies among particular species (Ewing and

Bennet-Clark 1968; Kyriacou and Hall 1980; Cowling and Burnet 1981; Cobb *et al.* 1989). Female *D. melanogaster* use the pulse song to identify conspecific males as well as to choose among particular males (Talyn and Dowse 2004).

Matings do occur between *D. melanogaster* and *D. simulans*, although laboratory studies have shown that few progeny are produced and that the hybrid offspring are infertile. Interestingly, *D. simulans* females rarely mate with *D. melanogaster* males, whereas *D. melanogaster* females mate much more readily with *D. simulans* males (Das *et al.* 1995; Moulin *et al.* 2004). This difference between the reciprocal crosses has been attributed partly to male and partly to female effects. Differences in cuticular hydrocarbon profiles and aspects of the male courtship song between these species seem to be likely candidates contributing to premating isolation (Moulin *et al.* 2004). Regardless, *D. melanogaster* males actively court *D. simulans* females, and *D. melanogaster* males recently rebuffed by a heterospecific female are less likely to court another such female, although they court conspecific females (Dukas 2004). This work suggests that *D. melanogaster* males learn to recognize the heterospecific females and avoid them (Dukas 2004). The learning process likely occurs via a rapid nervous system mediated response to pheromonal and behavioural cues from the female.

We investigated this process of choosing or learning to avoid potential mates in greater detail by examining the kinetics of *D. melanogaster* male courtship towards *D. melanogaster* or *D. simulans* females. The earlier observation by Dukas (2004) led us to ask how rapidly a *D. melanogaster* male learns to reduce courtship towards a heterospecific female, so we developed an assay that allowed us to determine the

timescale on which the learning process occurs. We found that sexually inexperienced *D. melanogaster* males rapidly decrease courtship towards *D. simulans* females within 20 min of an initial courtship exposure. Males show high levels of courtship towards heterospecifics for the first few minutes after encountering a female, but rapidly curtail courtship within 5 min.

Our lab previously showed that social interactions that occur when *D. melanogaster* males court conspecific females lead to measurable changes in transcript levels (Carney 2007). In this study, we asked if courtship interactions between a *D. melanogaster* male and a *D. simulans* female similarly lead to altered gene expression patterns. We found that nine genes have decreased expression in males that court heterospecific females. Of these nine genes, eight are also down regulated when males court conspecific females. The number of genes with altered expression solely in response to conspecific courtship is much higher (27 genes) than the number affected by both conspecific and heterospecific courtship (eight genes). Together, our behavioural and genomewide expression data indicate that the social interactions that occur during conspecific, but not heterospecific, courtship may affect reproductively important genetic cascades that are potentially involved in species recognition, premating reproductive isolation and speciation.

Materials and Methods

Courtship protocol

The laboratory strain, *D. melanogaster Canton-S* (*CS*) and the wild-caught *D.*

simulans Sim2 strain (established from flies collected in Marietta, GA, USA in 2003) were maintained at 25°C on a 12 hr light/dark cycle on a standard cornmeal, sugar and agar medium. *CS* virgin males and females and *Sim2* virgin females were collected within 1–2 hr of the initiation of the lighted phase of the light/dark cycle and were kept in yeasted vials in groups of 20 or fewer flies. On day 4, males were aspirated into individual vials. *CS* and *Sim2* female genitals were cauterized on day 4 to prevent mating, and groups of 20 females were placed in vials until the behavioural assays were performed the next day. On day 5, a single *CS* or *Sim2* female was aspirated into a male's vial within 2–4 hr of the beginning of the lighted phase of the cycle. All virgin collections and behavioural assays were performed during the same time window each day to control for circadian effects on gene expression patterns. Pairs of flies were watched for 20 min, and the presence or absence of courtship activity (following, wing extensions and attempted copulation) was assessed for each minute of the observation period to calculate the time each male spent performing courtship. Average total courtship time and average courtship per minute values were determined for males that courted conspecific or heterospecific females. Males courted *CS* females an average of 15.0 min (n=379, SE=4.62), whereas they courted *Sim2* females 5.7 min on average (n=347, SE=5); this courtship towards *D. simulans* occurred mostly within the first 5 min of exposure.

Statistical analysis

Each male was scored at aech minute of the 20-min observation time for the

presence or absence of courtship towards either a conspecific *D. melanogaster* female or a heterospecific *D. simulans* female. A two-tailed t-test comparison identified a significant (t_{724}=3.9, p<0.0001) increase in total courtship towards conspecific females compared with heterospecific females. Male courtship is dependent on the species of the female, the time throughout the observation, and the interaction between time and female [binary logistic regression, time ($Wald_1$=2913.3, SE=0.039, p<0.0001), female ($Wald_1$=522.2, SE=0.003, p<0.0001), interaction ($Wald_1$=2790.3, SE=0.077, p<0.0001).

Affymetrix microarrays

Sample collection, RNA extractions, cDNA preparation and microarray experiments discussed in this study were carried out concurrently with those described in a previously published study assessing the male *D. melanogaster* genome-wide response to conspecific courtship interactions (Carney 2007). This experimental designed allowed us to compare genome-wide responses to heterospecific and conspecific courtship interactions using a common set of control samples.

We collected virgin *CS* males and females and virgin *Sim2* females, aged them and performed courtship exposures as described previously (Carney 2007). Briefly, stock vials were maintained at 25^{o}C, and virgin flies were collected and aged at 25^{o}C in groups of 20 or fewer flies. On day 3, individual males were aspirated into new food vials. On day 4, a single female (either *CS* or *Sim2*) was aspirated into the experimental vials. Mock-exposed males served as the control flies for baseline levels of gene expression in our comparisons. These control males were treated the same way as males

that were placed with a female (aging, transfer), except that we used the aspirator to blow into the vial but did not transfer a female. After a 5-min courtship (or mock) exposure to either a *CS* or a *Sim2* female, male flies were quick-frozen and stored at -80^{o}C until RNA extractions were performed using Trizol reagent (Invitrogen, Carlsbad, CA, USA). All virgin collections, treatments and behavioural assays were performed at the same time of day to control for circadian effects on expression patterns.

Whole-animal total RNA was extracted from control males or those that courted *CS* or *Sim2* females. As described previously (Carney 2007), we made five RNA preparations, each from control or courtship-exposed males for a total of 15 sets of RNA, approximately 12 males per RNA preparation. The RNA preparations for all three treatment groups were labelled and hybridized to Affymetrix Drosophila Genome Arrays (Affymetrix version 1, based upon Berkeley Drosophila Genome Project v4.0; Santa Clara, CA, USA). Sample labelling and microarray hybridizations were carried out at the University of Kentucky MicroArray Core Facility using the standard Affymetrix protocols. Therefore, all samples were collected and hybridized using the same experimental conditions.

For microarray data analysis, we used the Mixed procedure (SAS Institute Inc., Cary, NC, USA), taking into account both the fixed treatment (mock, conspecific or heterospecific) variable and the random chip (1–5) variable, on expression values derived using three different methods: GCOS (GeneChip® Operating Software) (Affymetrix) and the PM-only and PM-MM methods from dChip (Li and Wong 2001). PM refers to a perfect match between the probe and Drosophila reference sequences,

whereas MM refers to a single nucleotide difference in the probe compared with the reference sequence that should prevent hybridization. In our analyses, we only considered probe sets with expression values greater than 50 for the dChip algorithms and greater than 100 for the GCOS algorithm.

The data set presented here showed significant ($p<0.05$) changes in expression for at least two of the three algorithms and had a false discovery rate less than 0.05 (Storey and Tibshirani 2003). Post-hoc analysis (Tukey's) identified genes differentially ($p<0.05$) expressed between treatments. Analyses of results comparing males that courted conspecific females to mock exposed males were previously published (Carney 2007). This earlier data set was reanalysed in the current study in order to incorporate the *D. simulans* data set and make comparisons among the three treatment groups.

qPCR

We designed primers specific to two down-regulated genes (*CG4757* and *IM23*) to use for independent validation of the microarray results. Both genes are down regulated in males that court conspecific females (Carney 2007) as well as in males that court heterospecific females (Table 1). Using the protocol described above for microarray experiments, we generated RNA preparations for courting and control males and used these samples to prepare cDNA for qPCR. Behavioural tests were performed and RNA was extracted for qPCR analysis at the same time as the samples that were used for array hybridization. Genes responsive to conspecific courtship were validated previously (Carney 2007). We no longer have samples appropriate for additional qPCR

validation, as the RNA preparations were exhausted during the qPCR analyses described in this study and in Carney (2007).

RNAextractions were performed with the Trizol reagent (Invitrogen) and cDNA was prepared following the standard protocol from the Superscript First-Strand Synthesis Kit (Invitrogen). cDNAs were diluted 1 : 10 for qPCR reactions and were amplified using the SYBR green mastermix for qPCR (Applied Biosystems, Foster City, CA, USA). PCR reactions were performed in the ABI7700 using default run parameters. Positive and negative control reactions were included on each plate and melting curve analyses were performed at the end of each run to test for primer specificity.

Control amplification reactions with *rp49* primers allowed us to normalize the amount of cDNA in each reaction. We used the Relative Standard Curve method (Applied Biosystems) which compares control (*rp49*) and experimental primer amplifications to determine relative mRNA concentrations for each sample.

Results

Courtship behaviour towards conspecific and heterospecific females

The work of Dukas (2004) indicated that *D. melanogaster* males learn to avoid heterospecific *D. simulans* females after unsuccessful attempts at mating. In this earlier study, males were placed with heterospecific females for 1 hr and then given an opportunity to court either a conspecific or heterospecific female. Males significantly reduced courtship towards the heterospecific but not the conspecific female (Dukas 2004). Given the 1 hr interaction period, it was unclear how rapidly learning can take

place. We investigated the kinetics of this response in greater detail using a 20 min courtship assay.

Since a sexually mature *D. melanogaster* male typically mates within 5–10 min of being placed with a mature *D. melanogaster* virgin female, we cauterized the genitals of both types of test females to prevent mating; this enabled us to evaluate courtship levels for a longer period of time. We paired sexually inexperienced *D. melanogaster* males with either a conspecific (*CS*) or heterospecific (*Sim2*) female for 20 min and assessed levels of courtship towards each type of female.

When a *D. melanogaster* male was paired with either a *D. melanogaster* or a *D. simulans* virgin female, the males initially exhibited strong courtship towards each type of female (Figure 4). However, over time the males decreased courtship towards the heterospecific female, whereas they maintained high levels of courtship towards the conspecific female. Indeed, after 20 min with the female, approximately 70% of the males paired with a *D. melanogaster* female were still courting, whereas only 13% of the males paired with a *D. simulans* female continued to court (Figure 4).

The majority (84.96%) of males presented with a *D. melanogaster* female spent more than 10 min courting the female, and greater than 12% of the males showed sustained courtship for the 20-min duration of the experiment. In contrast, few (19.88%) males provided with a *D. simulans* female spent more than 10 min performing courtship behaviours (Figure 5; average total courtship time towards *D. melanogaster* females=15.04 min; *D. simulans* females=5.73 min, $t_{724}=3.9$, $p<0.0001$). Together, these results indicate that *D. melanogaster* males exhibited sustained courtship towards

conspecific females, whereas their courtship towards heterospecific females decreased over time.

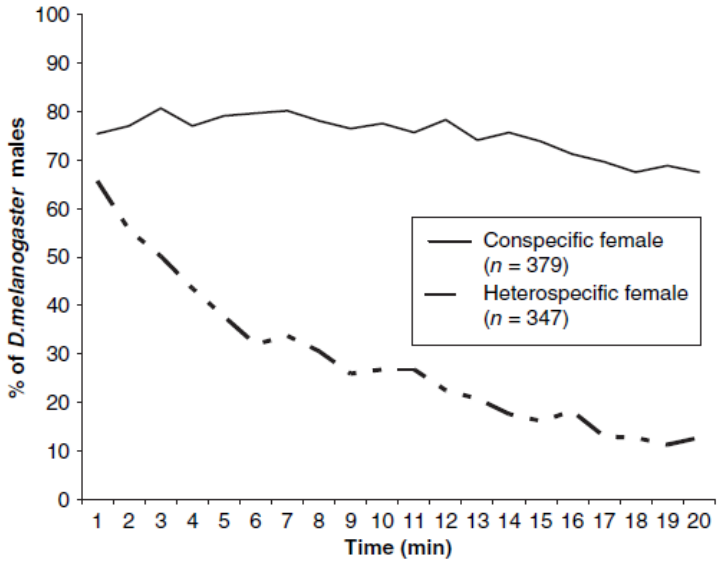


Figure 4. **Males rapidly decrease courtship towards heterospecific females.** Courtship interactions between *Drosophila melanogaster* males and either conspecific or heterospecific females were assayed. The presence of courtship behavior was assessed each minute of the 20 min observation window for each male tested. Males rapidly decreased courtship towards heterospecific females within 5 min of exposure.

Genome-wide response to courtship interactions

Our previous work showed that *D. melanogaster* males that court conspecific females have altered levels of gene expression compared with males that are mock-exposed to courtship (Carney 2007). There are a small number of genes whose transcripts levels are increased in *D. melanogaster* males that court *D. melanogaster* females, and at least two of these genes are downstream targets of the genetic hierarchy that regulates reproductive behaviours in both sexes. Interestingly, many of the

transcripts with decreased abundance in courting males encode products that function in the immune response (Carney 2007).

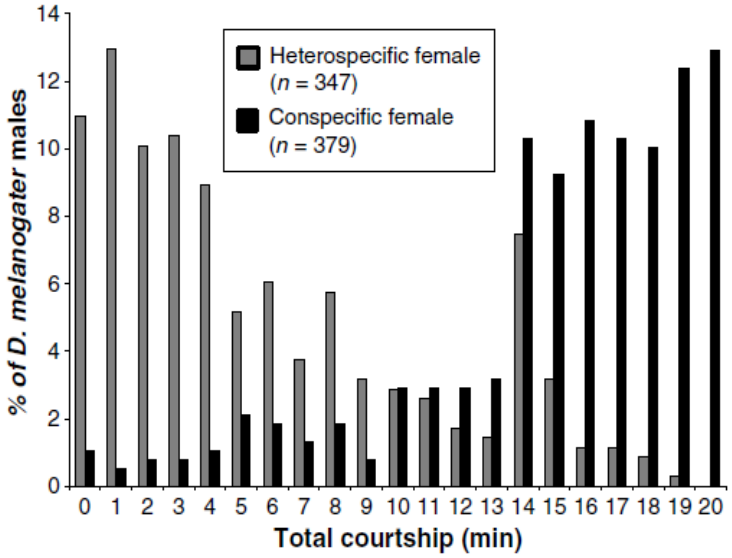


Figure 5. **Total courtship towards heterospecific females is decreased.** Overall courtship levels were decreased in *Drosophila melanogaster* males that courted *Drosophila simulans* females (grey bars) compared with *D. melanogaster* males that courted conspecific females (black bars). The majority of males spent less than 10 min courting heterospecific females but more than 10 min courtship conspecific females.

We expected that a genome-wide response to courtship would also occur in males that court heterospecific females, but that it would differ from the response to conspecifics. To test this hypothesis, we collected total RNA from *D. melanogaster* males that had either courted a *D. simulans* female or had been mock-exposed to courtship. These RNA preparations were labelled and hybridized to Affymetrix Drosophila genome arrays (see Materials and Methods). The experiments were carried out concurrently with earlier experiments that evaluated the genome-wide response to conspecific courtship (Carney 2007).

By analysing the expression values for the three treatments – males that courted *D. melanogaster* females, males that courted *D. simulans* females and males that were mock-exposed to courtship – we found that eight transcripts are affected by conspecific as well as heterospecific courtship interactions (Table 1), a number that is much smaller than those whose abundance changes specifically because of conspecific courtship (Table 2); all eight transcripts have reduced expression in courting males compared with control males. Interestingly, expression of a single gene, *CG1857*, is specifically decreased during heterospecific courtship. *CG1857* is predicted to encode a serine-type endopeptidase inhibitor that likely functions in the immune response.

Table 1. Genes down regulated in males that court either a conspecific or heterospecific female.

Annotation symbol	Gene symbol	Location	Molecular function	Biological process	Immune function
CG6639		36C9	Serine-type endopeptidase activity	Proteolysis	Y
CG16772		38A8	Unknown	Unknown	Y
CG18279	*IM10*	50A5	Unknown	Toll signalling pathway; antibacterial humoral response; defence response	Y
CG15066	*IM23*	55C4	Unknown	Toll signalling pathway; antibacterial humoral response; defence response	Y
CG18108	*IM1*	55C4	Unknown	Defence response	Y
CG15065		55C4	Unknown	Unknown	Y
CG16836		55C4	Unknown	Unknown	Y
CG4757		85D5	Carboxylesterase activity	Unknown	Y

In our original assessment of conspecific courtship interactions, we identified 43

transcripts whose levels are courtship responsive (Carney 2007). We reanalysed this earlier data in the current study and identified 35 genes that are down-regulated; 27 of these genes are down regulated specifically in males that court conspecific females (Tables 1 and 2). Therefore, conspecific courtship causes expression level changes in a much larger set of gene products than heterospecific courtship.

Similarly to our earlier study characterizing the genome-wide response to conspecific females, we found that the eight genes that are common to males that court both types of female have been implicated as functioning in the immune response (Table 1). Eight of the 27 genes down regulated because of conspecific courtship are predicted to function in immune signalling as well (Table 2). In contrast to our earlier work, we did not identify any gene products with significantly increased levels in males that court either type of female.

qPCR validation of microarrays

We used qPCR to assess the transcript levels for *CG4757* and *IM23* in males who courted heterospecific females relative to control males. Both genes were downregulated during conspecific courtship (Carney 2007). We found in each case that message levels are decreased in *D. melanogaster* males that courted *D. simulans* females (Table 1), and the differences were statistically significant (t_8=2.7, $p<0.05$) for CG4757. Conspecific courtship responsive genes were validated previously (Carney 2007).

Table 2. Genes down regulated only in males that court a conspecific female

Annotation symbol	Gene symbol	Location	Molecular function	Biological process	Immune function
CG13384		29B4-29C1	Amino acid transmembrane transporter activity	Amino acid transport	Y
CG3937	*cher*	89E12-89E13	Actin binding	Ovarian ring canal formation; protein localization; ring canal formation, actin assembly; germarium-derived female germ-line cyst encapsulation; ring canal formation; determination of adult life span; learning or memory; olfactory learning; negative regulation of lamellocyte differentiation	Y
CG1744	*chp*	100B5-100B6	Protein binding	Homophilic cell adhesion; compound eye photoreceptor development; rhabdomere development	
CG7766		8C8-8C9	Phosphorylase kinase regulator activity; catalytic activity; calmodulin binding	Glycogen metabolic process	
CG2943		84E4-84E5	Unknown	Unknown	
CG12351	*deltaTry*	47F5	Serine-type endopeptidase activity	Proteolysis	Y
CG10810	*Drs*	63D2	Unknown	Defence response to fungus; defence response; innate immune response; antifungal humoral response; defence response to Gram-negative bacterium; antibacterial humoral response	Y
CG18106	*IM2*	55C4	Unknown	Defence response	Y
CG9057	*Lsd-2*	13A8-13A9	Protein binding	Lipid storage; sequestering of triglyceride; lipid particle transport along microtubule; regulation of transport; lipid metabolic process	
CG4577		21F1	Unknown	Unknown	
CG31956	*pgant4*	23F3	Polypeptide N-acetylgalactosaminyltransferase activity	Unknown	
CG17633		30C8	Metallocarboxypeptidase activity; zinc ion binding	Proteolysis	
CG1553		43E17-43E18	Unknown	Unknown	
CG13482		70D5	Unknown	Unknown	Y

Table 2. Continued

Annotation symbol	Gene symbol	Location	Molecular function	Biological process	Immune function
CG4562		92B4	ATPase activity, coupled to transmembrane movement of substances; transporter activity; ATP binding	Transport	
CG6277		97D14	Triacylglycerol lipase activity; phospholipase A1 activity	Lipid metabolic process	
CG5791		94A2	Unknown	Unknown	Y
CG15231	*IM4*	57B3	Unknown	Defence response; immune response	Y
CR32477	*CR32477*	61B2	Unknown	Unknown	
CG5237	*unc79*	91F12	Protein binding; zinc ion binding	Unknown	
CG31004		100B5	Unknown	Cell-matrix adhesion	
CG33519	*Unc-89*	60B1-60B2	ATP binding; Rho guanyl-nucleotide exchange factor activity; protein serine/threonine kinase activity	Protein amino acid phosphorylation; regulation of Rho protein signal transduction	
CG4841		36B4	Unknown	Unknown	
CG12085	*pUf68*	62A3	Poly(U) binding; mRNA binding; nucleotide binding	Nuclear mRNA splicing, via spliceosome; alternative nuclear mRNA splicing, via spliceosome; cystoblast division; mRNA splice site selection; regulation of alternative nuclear mRNA splicing, via spliceosome; regulation of cell cycle	
CG5823		90B3	Ubiquitin-protein ligase activity	Regulation of protein metabolic process; post-translational protein modification	
CG6386	*ball*	97D3	Protein kinase activity; histone threonine kinase activity; protein serine/threonine kinase activity; ATP binding	Protein amino acid phosphorylation; histone phosphorylation; peptidyl-threonine phosphorylation; female meiosis; karyosome formation	
CG9086		15C6	Ubiquitin-protein ligase activity; protein binding; zinc ion binding	Protein ubiquitination; protein catabolic process	

Discussion

When fruit flies encounter one another in the wild, they need to make rapid determination of the appropriateness of the second individual as a mate, because valuable energetic resources will be wasted on heterospecific matings. Flies use a variety of sensory cues to aid them in making this choice. The sensory information must be received and evaluated by the nervous system to indicate to the animal whether or not the interaction is likely to be fruitful. In most cases, this process probably relies upon rapid nervous system function. However, our genome-wide analysis of Drosophila conspecific and heterospecific courtship interactions suggests that a second slightly delayed mechanism may also come into play.

In a 10-min courtship test, sexually inexperienced *D. melanogaster* males court *D. melanogaster* and *D. simulans* females to a similar extent (Dukas 2004). However, when males who previously were placed with *D. simulans* females for 1 hr were then immediately presented with a new *D. melanogaster* or *D. simulans* female, the males decreased courtship towards the heterospecific female. Sexual experience with a heterospecific female did not reduce conspecific courtship levels. This result suggests that the *D. melanogaster* males learned to avoid heterospecific females (Dukas 2004).

Matings between these species produce infertile hybrids and are disadvantageous to both species, so we wondered how quickly the learning process takes place. Our results indicate that sexually naïve male *D. melanogaster* males initially court both conspecific and heterospecific females at high levels (Figure 4). However, they reduce courtship towards heterospecifics over time (Figure 4), and only 19.88% of males spent

more than 10-min courting a heterospecific female (Figure 5). In contrast, 84.96% of males court a conspecific female for more than 10 min (Figure 5). These results show that the males learn extremely rapidly (within a matter of minutes) to reduce courtship towards a heterospecific female. Interestingly, they continue to court conspecific females at a very high level. Therefore, the reduced courtship towards the heterospecific female is likely a male response to a pheromonal or behavioural cue from this female that is different from the cue relayed by a conspecific female.

An earlier study from our lab showed that *D. melanogaster* males have a rapid genome-wide response to courtship directed towards conspecific females (Carney 2007). In whole animals, a small number of genes, including downstream components of the genetic hierarchy that establishes the potential for reproductive behaviour, have increased message levels in males that court conspecific females. These expression changes are unlikely to be due solely to movement of the fly as mock-exposed and courtship-exposed flies have similar motility in our assays (L. L. Ellis & G. E. Carney, unpublished results) and these same genes do not have increased expression in males that actively court heterospecific females.

An interesting result from our new comparison of gene expression profiles in control males vs. those that have courted a *D. simulans* female is that there are no transcripts with increased expression in males that court heterospecific females; there are also fewer transcripts with decreased levels in males that court heterospecifics (9 transcripts) compared with those that court conspecifics (35 transcripts). These results suggest that there is a signalling mechanism responsive to conspecific courtship that in

turn leads to changes in message levels.

We did not identify any genes that are up regulated by courtship in this study using a significance cutoff of $p<0.05$. However, one gene, *bubblegum* (*bgm*), is significantly up regulated because of conspecific courtship at $p=0.05$. This result is intriguing because other studies in our lab indicate that *bgm* is up regulated in mated *bgm* levels may begin to rise during courtship and are either maintained or are augmented by mating.

In the current study, we reanalysed data from our previous work (Carney 2007) to allow us to compare across the three treatment groups. Although we see the same trends in the conspecific courtship results (i.e. more transcripts have decreased levels and many function in the immune response), the identity of the genes from the new analysis differs somewhat (10／35 genes overlap). This discrepancy is likely because of differences in the data analysis techniques employed in the two studies. In our new study, we took a conservative approach to identifying responsive genes by incorporating a false discovery rate parameter (Storey and Tibshirani 2003), using a Mixed model ANOVA, and requiring significance in at least two of the analyses of expression values. If we relax these criteria to allow genes to be placed in the list that only are identified by one analysis, the majority of genes up regulated in response to conspecific courtship and qPCR validated in Carney (2007) are also found to be responsive to conspecific (but not heterospecific) courtship in this study. Additional down-regulated genes from the earlier study are also present using this less conservative treatment of the data.

Work in our lab indicates that at least some of the genes that are identified

through this type of genomic profiling approach have important functions in reproductive behaviour. For example, mutations in *egghead* (*egh*), a gene whose expression increases in male brains during conspecific courtship, significantly reduce male-to-female courtship levels (L. L. Ellis & G. E. Carney, unpublished data). A second courtship responsive gene, *female-specific independent of transformer* (*fit*), is a downstream component of the genetic hierarchy that regulates fly reproductive behaviours. Therefore, this gene is also likely to have important functions in behaviour (Carney 2007).

In addition to the rapid assessment that occurs via the nervous system when organisms interact, there also appears to be a secondary response that occurs. Genes are either up or down regulated in response to the social environment of the animal, and these gene products possibly modify behaviours. Positive pheromonal or behavioural cues from conspecific females may activate this signalling response, whereas negative cues or a failure to respond to cues from heterospecific females may not allow full activation of the response.

Gene expression changes because of social interactions have been examined in birds and mammals to the greatest extent (Hughes and Dragunow 1995; Ball and Balthazart 2001; Mello 2004; Mello *et al.* 2004; Pinaud 2004; Bradley *et al.* 2005), although other species are being examined (Ben-Shahar *et al.* 2002; Burmeister *et al.* 2005). In vertebrates, immediate early genes are induced when animal cells encounter hormones, growth factors or other stimuli in their environment (Hughes and Dragunow 1995). Birds rapidly induce ZENK expression in response to a song stimulus (Mello *et*

al. 1992; Mello and Clayton 1994) or by engaging in a behaviour (Jarvis and Nottebohm 1997). Mammals also respond genetically to their environment (Hughes and Dragunow 1995; Pinaud 2004), and alternative bee social behaviours correlate with gene expression changes (Ben-Shahar *et al.* 2002; Sinha *et al.* 2006). Many immediate early genes encode transcription factors predicted to regulate expression of a second set of genes that, in turn, modulate plasticity (Hughes and Dragunow 1995). Changes in DNA methylation (Champagne and Curley 2005) and neuronal components likely lead to long-term changes in central nervous system gene expression, function and connectivity (Kozorovitskiy *et al.* 2006).

Our work shows that gene expression patterns rapidly change because of social cues in *D. melanogaster* as well. This work suggests that these rapid changes may serve as a secondary mechanism for activating genes that function in reproduction. We anticipate that some loci identified by these types of studies encode gene products that have modulatory function in the nervous system. Microarray analysis alone is not sufficient to completely characterize the full suite of genes involved in these responses, however, as some genes may be expressed at low levels or in small numbers of cells. In addition, these studies do not allow one to identify mechanisms that function independently of transcriptional activation or message stability. The gene expression results from this type of study do provide a starting point for further characterization of pathways, but ultimately molecular biological and genetic approaches will be necessary.

Our results may be especially interesting with respect to reproductive isolation and speciation. If there are genetic cascades that must be activated for mating to be

successful, then genes in these pathways could be involved in generating reproductive barriers among distinct gene pools. In geographically isolated populations, for example, divergence of these loci, as a consequence of genetic drift or sexual selection, could conceivably contribute to allopatric speciation. In sympatric populations, such loci could be the targets of reinforcement resulting from selection against genetically unfavourable hybridization events. Regardless, future characterization of the genetic pathways activated during courtship and mating should generate insights into a number of key evolutionary processes such as species recognition, sexual selection, reinforcement and speciation.

CHAPTER III

THE COURTSHIP-RESPONSIVE GENE *egghead* IS REQUIRED IN *apterous* NEURONS FOR *Drosophila melanogaster* MALE COURTSHIP BEHAVIOR

Introduction

Behaviors are complex processes resulting from an organism's ability to integrate sensory cues into physiological and motor outputs. Adding to the complexity of this process are the effects from the organism's genetics and environment, including social interactions, on behavior, brain morphology, and gene expression (Siegel and Hall 1979; Levine *et al.* 2002; Shen *et al.* 2004; Stewart and McLean 2004; Burmeister *et al.* 2005; Kozorovitskiy *et al.* 2006; Yurkovic *et al.* 2006; Carney 2007; Technau 2007; Ellis and Carney 2009).

It is possible to use microarray technology to assess changes in mRNA expression occurring during or in response to behavior as a means to gain insight into physiological changes that also occur. Several studies, particularly in songbirds, bees and fruit flies, have examined transcript level changes in freely behaving animals. In songbirds, 33 genes are regulated by singing behavior, including loci involved in signal transduction and synaptic signaling (Wada *et al.* 2006), and a variety of social environments and stimuli impact honey bee brain gene expression (Grozinger *et al.* 2003; Whitfield *et al.* 2003; Whitfield *et al.* 2006; Sen Sarma *et al.* 2009). Similarly, male *Drosophila melanogaster* show rapid changes in transcript levels due to courtship interactions with females (Carney 2007; Ellis and Carney 2009). Though the signaling

cascades mediating changes in mRNA levels due to behavior and social interactions are unclear, by studying these changes we can clarify the intracellular processes affecting nervous system function, physiology and behavior. An advantage of such studies in Drosophila is that mutant strategies can be employed to characterize behavioral requirements for responsive loci.

The courtship behaviors of male Drosophila are influenced by genetics (Billeter *et al.* 2002) and social interactions (Ewing 1983; reviewed in Greenspan and Ferveur 2000; Mehren *et al.* 2004). The somatic sex-determination pathway regulates these behaviors (Cline 2005; Shirangi and McKeown 2007) and the sexually dimorphic development of Drosophila, including that of the nervous system (Finley *et al.* 1997; Kimura *et al.* 2005; Manoli *et al.* 2005; Stockinger *et al.* 2005; Rideout *et al.* 2007; Sanders and Arbeitman 2008; Mellert *et al.* 2010; Rideout *et al.* 2010; reviewed in Billeter *et al.* 2006). Though target loci of the transcriptional regulatory members of this pathway are known (Burtis *et al.* 1991; Cann *et al.* 2000; Kopp *et al.* 2000; Dauwalder *et al.* 2002; Drapeau *et al.* 2003; Fujii and Amrein 2002; Arbeitman *et al.* 2004; Goldman and Arbeitman 2007; Lazareva *et al.* 2007; Fujii *et al.* 2008; Dalton *et al.* 2009), few have clearly defined functions in behavior and neural development. Several elegant Drosophila microarray studies were key to identifying most of these downstream targets (Arbeitman *et al.* 2004; Goldman and Arbeitman 2007; Dalton *et al.* 2009), but the strategies used do not allow us to distinguish target genes that affect development of the nervous system from those that impact physiology and behavior post-development.

During courtship, males are exposed to sensory information that must be rapidly

interpreted to create the appropriate behavioral response (*e.g.*, continue courtship directed toward that fly or seek a new mate). In males, courtship causes rapid expression level changes detectable in whole animals (Carney 2007; Ellis and Carney 2009). These rapid responses are likely mediated by signaling in the nervous system, sensory organs and other tissues that affect neural physiology. Our approach has the advantage of using wild-type animals performing behaviors to identify adult-expressed gene products that are impacted by behavior, including target genes of the somatic sex-determination hierarchy.

In our current study we focused on gene expression changes occurring in the male head (rather than whole body) during courtship toward a female. We also expanded on our earlier studies by showing that courtship-responsive loci can function in behavior. Our data indicate that courtship causes gene expression changes in loci expressed in neuronal as well as non-neuronal tissues that may modulate neural signaling and behavior. At least 3 identified genes are downstream components of the sex-determination hierarchy. Using available mutations in the courtship-responsive gene *egh*, we found that adult *egh* expression is important for robust male-to-female courtship. *egh* expression in Ap neurons is sufficient to restore proper courtship behavior, indicating that *egh* expression in the Ap circuit is important for male reproductive behavior.

Materials and Methods

Microarray analysis

We used an isogenized wild-type *Canton-S (CS)* strain and handled flies similarly to Carney (2007) except that the females' genitals were electrically cauterized to prevent mating (non-mateable females). Twenty or fewer virgin isogenic *CS* males were aged collectively for 3 days, and 20 or fewer virgin isogenic *CS* females were aged collectively for 3 days. On day 4, males were aspirated into individual vials, and females had their genitals cauterized by passing a 4 mA current over 2 fine tungsten wires on the external genitalia of the female to prevent mating. Females were placed in a new vial and given one day to recover. All flies were kept on a 12-hr light/dark cycle at 25^{o}C, and we performed all procedures within 2 hrs of lights on to control for circadian effects on gene expression and behavior.

Analysis of courtship behavior on day five included equally dividing males into two groups: (1) Control and (2) Courting males. For the courting male treatment, one cauterized female was aspirated into a male's vial. Control males were treated the same except that a female was not transferred during the aspiration process. Courtship exposure lasted for 20 min and the presence of courtship was assessed at 1 min intervals. Only males that courted at least 70% of the time were collected for analysis. After 20 min, the males were removed from the vials, quick frozen in liquid Nitrogen, and stored at -80^{o}C for future RNA extraction.

By vortexing quick-frozen flies, we separated heads from the rest of the bodies. For each treatment, 20 male heads were randomly assigned to one of 10 groups, giving

us 10 RNA preparations each for control and courting males. Total head RNA was extracted using Trizol (Invitrogen, Carlsbad, CA, USA) following standard protocols. The University of Kentucky MicroArray Core Facility labeled and hybridized 5 control and 5 courting male head RNA preparations to Affymetrix Drosophila 2.0 Genome Arrays following standard Affymetrix (Santa Clara, CA, USA) protocols.

We extracted expression values from the microarrays and conducted Bayesian *t*-test (CyberT, Baldi and Long 2001) and false discovery rate analyses ($q<0.05$, Storey and Tibshirani 2003) as described previously (Ellis and Carney 2009). Five algorithms, GeneChip® Operating Software (GCOS) (MAS 5.0, Affymetrix, Santa Clara, CA, USA), Gene Spring (Agilent, Santa Clara, CA, USA), PM and PM-MM (dChip, Li and Wong 2001) , and GCRMA (R, R Development Core Team) were used. We used the same statistical cut-off values from our previous work (Ellis and Carney 2009) to determine significantly ($p<0.001$ in 3 of 5 algorithms) up- and down-regulated courtship-responsive genes. Over-representation of gene ontology molecular functions or biological processes was determined using Fisher's exact test as stated previously (Carney 2007; Ellis and Carney 2009).

q PCR

We confirmed the microarray results by qPCR analysis on 5 control and 5 courting male RNA preparations that were not used for microarray hybridization. cDNA was synthesized from poly^{+}A purified (Oligotex mRNA mini kit, Qiagen, Netherlands) RNA using the SuperScript First-Strand Synthesis System (Invitrogen, Carlsbad, CA,

USA). Primers were designed for a randomly chosen set of 6 up-regulated (*CG9377, CG10621, egh, HLHmβ, Lsp2, sug*) and 3 down-regulated (*CG31181, Rim, Sh*) candidate genes predicted to be enriched in the brain, fat body or both tissues. Genes with low predicted transcript levels in the head were not tested (Chintapalli *et al.* 2007). To control for amplification specificity primer pairs were designed across introns when possible. No template controls as well as controls with template but without Reverse Transcriptase were included in the analysis.

Using the ABI7500 and its default parameters (Applied Biosystems, Foster City, CA, USA), each template was run in triplicate, using 2 μL of a 1:4 cDNA dilution and the SYBR Green PCR Mastermix (Applied Biosystems, Foster City, CA, USA). We used dissociation curve analysis to determine primer-specific amplification and the Relative Standard Curve Method (Applied Biosystems, Foster City, CA, USA) to determine transcript levels. Normalization to *rp49* levels generated relative transcript abundance values for control or courting male samples. The relative fold change for each gene was measured as the ratio of courting male relative abundance to control male relative abundance, and significance was determined by a two-tailed *t*-test. Up regulation of *egh* and *HLHmβ* and down regulation of *CG31181* were confirmed by secondary qPCR analysis.

In situ hybridization

We performed *in situ* hybridization for a subset of courtship-responsive genes. Genes and their corresponding cDNA clones were *CG9377* (GH08193), *CG10621*

(RE64786), *cwo* (LD15411), *egh* (GH01085), and *sug* (LD36528). Antisense and sense probes were made from the above clones using the Digoxigenin (DIG)-labeling kit's standard protocol (Roche, Nutley, NJ, USA). Probes were hydrolyzed into 200 bp fragments and hybridized to dissected male tissues, including the brain, head carcass, or abdominal cuticle as previously described (Arbeitman *et al.* 2004).

Courtship behavior analysis

Flies were maintained on a 12-hr light/dark cycle at 25°C, except when noted otherwise. The Bloomington Stock Center supplied P-element insertion mutants (egh^{EP804}, $egh^{EY03917}$). Both insertions are located within the first *egh* exon. *egh* is located on the X chromosome, so males are hemizygous for *egh* alleles. For both X-linked P-element insertions, we crossed P-element females to isogenic *CS* males, and we crossed P-element males to isogenic *CS* females, generating experimental and control males, respectively, in genetically similar backgrounds. For behavioral analysis, P-element and control males were aged at 25°C in individual vials for 4 to 5 days and *CS* virgin females (20 or fewer) were aged collectively for 3 to 5 days. All courtship tests with *egh* mutants were performed in dim, red light conditions because mutations in *egh* affect neuron pathfinding in the visual system (Fan *et al.* 2005) and are therefore likely to impact eye function. In red light conditions, flies rely more heavily upon sensory systems other than the eye for courtship.

We analyzed courtship behavior under red light at 22°C and recorded the interactions with a digital camcorder. To analyze courtship behavior, a male was

aspirated into a mating chamber (diameter=1 cm) and a virgin *CS* female was introduced 2 min later. The pair was video recorded for 10 min. The courtship index (CI; percent of time the male spent performing courtship during the initial 10 min of observation) was calculated. CI values were arcsine transformed and two-tailed *t*-test comparisons between mutants and controls were calculated to determine significance ($p<0.05$).

To reduce *egh* specifically in the adult nervous system we utilized two *egh*-RNA interference (RNAi) alleles, egh^{v45160} and egh^{v45161}, from the Vienna Drosophila RNAi Center (VDRC) (Dietzl *et al.* 2007). We used *in situ* hybridization to verify reduced *egh* expression upon activation of each RNAi allele.

We targeted *egh* reduction pan-neuronally with $elav^{c155}$*-Gal4* (Lin and Goodman 1994) and more specifically with ap^{md544}*-Gal4* (Calleja *et al.* 1996) , which is expressed in *ap*-expressing neurons in larval and adult nervous systems. We increased the efficiency of the RNAi process by adding one copy of *UAS-Dicer-2* (VDRC). To reduce *egh* specifically in adults, the RNAi alleles were under the control of *UAS-tubulin-*$Gal80^{ts}$ (McGuire *et al.* 2004). Crosses were maintained at the permissive temperature of 20°C. Control males had *UAS-Dicer-2* and *UAS-tubulin-*$Gal80^{ts}$ and either the RNAi allele or the Gal4 driver. We collected virgin males and stored them in individual vials at either 20°C or 29°C. The courtship objects, *CS* virgin females, were collected and stored collectively at 25°C. Behavioral analysis was conducted under red light at the aforementioned temperatures. We used ANOVA and Tukey's post-hoc analysis to determine significant changes in CI due to temperature and genotype.

To restore *egh* expression, we crossed a genomic rescue construct (*eghP2*)

(Soller *et al.* 2006) to $egh^{EY03917}$. We used the above courtship assay (22°C; red light) to compare CIs of $egh^{EY03917}$*; eghP2* males to $egh^{EY03917}$ males collected during the same time frame. To narrow down which cells require *egh* expression for proper courtship behavior we utilized the rescue construct, *UAS-eghHA* (Soller *et al.* 2006). We crossed *UAS-eghHA* to the ap^{md544}*-Gal4* driver in the $egh^{EY03917}$ background and tested courtship activity at 22°C under red light. $egh^{EY03917}$ males with either component of the Gal4/UAS system served as controls.

Antibody staining

ap^{md544}*-Gal4* flies were crossed to flies containing a *UAS-GFPnls* allele. Adult males and females carrying both the Gal4 and UAS alleles were collected. Brains and VNCs were dissected in PBS, fixed in 4% paraformaldehyde and washed in PBS and PBST. We used a 1:50 concentration of anti-GFP in an overnight incubation. After more PBST washes, a 1:1200 concentration of secondary antibody was used.

Results

Male gene expression changes during courtship interactions

Within 5 min of male-to-female courtship, whole-animal transcript profiles are altered in courting males (Carney 2007; Ellis and Carney 2009). Next, we focused solely on male head gene expression in response to courtship since the head contains the brain as well as other tissues and sensory organs that impact behavioral and physiological responses to sensory inputs. We extended the courtship interaction period to 20 min to

ensure a robust response and used Affymetrix Drosophila 2.0 Genome Arrays to examine approximately 18,500 transcripts for expression level changes in males performing courtship toward non-mateable females (referred to as "courting males") compared to males that were not given a female courtship object ("control males") (See Materials and Methods).

Bayesian CyberT analysis comparing expression values from heads of courting males to those from controls identified 35 courtship-responsive loci (See Materials and Methods). Sixteen transcripts were up regulated (Table 3) and 19 were down regulated (Table 4) after 20 min of courtship. These changes are not likely due to locomotor differences since courting and control males have similar activity levels during the assay period (two-tailed *t*-test; $p>0.05$). Analysis by Fisher's exact test showed that several Gene Ontology annotations (molecular functions and biological processes) were over-represented in our data set compared to the Drosophila genome (Tables 3 and 4; $p<0.05$).

To verify our microarray results, we used qPCR to analyze transcript levels of candidate courtship-responsive genes in control and courting male head RNA preparations not used in the microarray study. The 6 up-regulated and 3 down-regulated courtship-responsive genes tested showed the expected trends in expression (Table 5).

Courtship-responsive genes are expressed in the brain and other head tissues

Because we assayed head tissue, identified loci may be expressed in the brain, sensory structures, the fat body, or a combination of these tissues. Expression of the

majority of courtship-responsive genes is enriched in the head relative to the brain, indicating higher expression in tissues outside of the brain (Chintapalli *et al.* 2007). Though some courtship-responsive genes are enriched in the eye, others are enriched in head tissues other than the brain or eye, including the adipose tissue lining the brain (Table 6).

Table 3. Candidate genes up regulated after 20 min of courtship

Gene identifier	Gene name	Avg. fold change	GO Molecular function	GO Biological process
CG1897	*Drop* (*Dr*)	1.46	Sequence-specific DNA binding	Nervous system development
CG3850	*sugarbabe* (*sug*)	1.6	Zinc ion binding	Regulation of transcription*
CG6494	*hairy* (*h*)	1.52	Transcription repressor activity*	Nervous system development*
CG6806	*Larval serum protein 2* (*Lsp2*)	1.34	Nutrient reservoir activity	Transport
CG9377		1.7	Serine-type endopeptidase activity	Proteolysis
CG9659	*egghead* (*egh*)	1.36	β-1,4-mannosyl-transferase activity	Axon guidance & oogenesis
CG9837		1.34	Unknown	Unknown
CG10142	*Ance-5*	1.34	Metallopeptidase activity	Proteolysis
CG10184		1.28	Pyridoxal phosphate binding	Amino acid metabolism*
CG10621		1.44	Homocysteine S-methyltransferase activity	Unknown
CG10812	*drosomycin-5* (*dro5*)	1.34	Unknown	Defense response to fungus
CG14489	*olf186-M*	1.26	Unknown	Unknown
CG14548	*E(spl) region transcript mβ* (*HLHmβ*)	1.58	Transcription repressor activity*	Nervous system development*
CG14688		1.28	Unknown	Unknown
CG17100	*clockwork orange* (*cwo*)	1.45	Transcription repressor activity*	Regulation of circadian rhythm

Table 3. Continued

Gene identifier	Gene name	Avg. fold change	GO Molecular function	GO Biological process
CG17820	*female-specific independent of transformer* (*fit*)	1.38	Unknown	Unknown

Comparing control male heads to courting male heads revealed that 16 genes are significantly ($p<0.001$) up regulated in male heads after 20 min of courtship. Over-represented molecular functions and biological processes (*$p<0.05$) were determined by Fisher's exact test.

Table 4. Candidate genes down regulated after 20 min of courtship

Gene identifier	Gene name	Avg. fold change	GO Molecular function	GO Biological process
CG1522	*cacophony* (*cac*)	-3.08	Voltage-gated calcium channel activity*	Courtship behavior
CG2217		-1.36	Unknown	Unknown
CG3738	*Cyclin-dependent kinase subunit 30A* (*Cks30A*)	-1.36	Cyclin-dependent protein kinase regulator activity	Cyclin catabolic process
CG4269		-3.92	Unknown	Unknown
CG9266		-1.28	Unknown	Unknown
CG9983	*hnRNA-binding protein 1* (*Hrb98DE*)	-1.24	Nucleic acid binding*	Unknown
CG10077		-1.3	ATP-dependent helicase activity	Unknown
CG10851	*B52*	-1.3	Nucleic acid binding*	Regulation of nuclear mRNA splicing, via spliceosome
CG12052	*longitudinals lacking* (*lola*)	-1.46	Zinc ion binding*	Brain morphogenesis
CG12295	*straightjacket* (*stj*)	-1.3	Voltage-gated calcium channel activity*	Synaptic vesicle fusion to presynaptic membrane
CG12348	*Shaker* (*Sh*)	-1.32	Voltage-gated cation channel activity*	Regulation of synaptic activity and courtship behavior*
CG12478	*bruno-3* (*bru-3*)	-1.26	RNA binding*	Negative regulation of translation
CG14755	*pou domain motif 3* (*pdm3*)	-1.38	Unknown	Unknown
CG31181		-1.36	Unknown	Unknown
CG31182		-1.44	Unknown	Unknown
CG33197	*muscleblind* (*mbl*)	-1.44	Zinc ion binding*	Muscle development
CG33547	*Rim*	-1.44	Small GTPase regulator activity	Regulation of exocytosis

Table 4. Continued

Gene identifier	Gene name	Avg. fold change	GO Molecular function	GO Biological process
CG42492		-1.34	Unknown	Unknown
CG42670	*pasilla* (*ps*)	-1.34	Unknown	Unknown

Average fold changes, molecular functions and biological processes are shown for 19 genes that are signficantly ($p<0.001$) down regulated in male heads after 20 min of courtship. *$p<0.05$, Fisher's exact test.

Table 5. qPCR confirmation of the microarray results

Gene identifier	Gene symbol	Relative fold change±SEM	Avg. relative expression level in control male heads±SEM	Avg. relative expression level in courting male heads±SEM
CG9377		1.25±0.25	0.07±0.01	0.09±0.02
CG10621		1.31±0.28	1.95±0.46	2.42±0.63
CG9659	*egh*	2.04±0.42	3.2±1.07	6.62±1.72
CG14548	*HLHmβ*	3.13±0.82*	0.65±0.16	1.92±0.62
CG6806	*Lsp2*	2.79±1.09	0.57±0.17	1.5±0.72
CG3850	*sug*	2.36±0.58*	3.95±1.2	9.31±2.58
CG31181		-2.7±0.09*	0.6±0.2	0.15±0.11
CG33547	*Rim*	-12.5±0.006	0.4±0.09	0.03±0.004
CG12348	*Sh*	-3.13±0.09*	0.39±0.09	0.12±0.04

Indicates a significant ($p<0.05$) difference in the average relative expression level in control male heads compared to courting male heads. SEM=Standard error of the mean.

Table 6. Courtship-responsive genes are enriched in head tissues including the brain and fat body

	Total no. of genes	Head	Brain	Eye	Fat body
Up regulated	16	14	6	11	12
Down regulated	19	17	17	16	15

Data was compiled from FlyAtlas (Chintapalli *et al.* 2007).

Two courtship-responsive genes, *Larval serum protein 2* (*Lsp2*) and *fit* are expressed in adipose tissue, also known as the fat body, surrounding the brain in both sexes (Benes *et al.* 1990; Fujii and Amrein 2002). *fit* was named due to its high level of expression in females compared to males and because its expression is regulated by the somatic sex-determination hierarchy gene *Sex-lethal* (Fujii and Amrein 2002). Though expression is low in virgin males, *fit* expression increases in response to courtship (Table 3) (Carney 2007). *In situ* hybridization confirmed that other courtship-responsive genes are expressed in the male fat body (*CG10621, sugarbabe* (*sug*)), the male brain (*CG9377*, *egh*), or both tissues (*clockwork orange* (*cwo*)) (Figures 6 and 7).

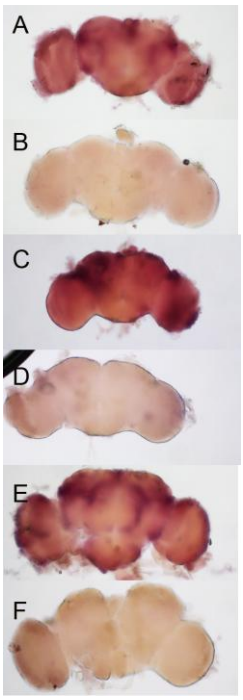


Figure 6. Courtship-responsive genes *CG9377, cwo,* and *egh* are expressed in the male brain. Antisense (A,C,E) or sense (B,D,F) RNA probes were designed to cDNA clones for *CG9377* (A,B), *cwo* (C,D), and *egh* (E,F). *In situ* hybridization to whole-mounted male *CS* tissue reveals that courtship-responsive genes are expressed in male brains.

egghead is required in the adult male brain for robust courtship

We hypothesized that courtship-responsive genes likely modulate courtship behavior, either by regulating the performance of courtship steps or by making the male a more efficient courter by increasing the efficiency of stimulus processing. This efficiency could affect the current courtship interaction or, more likely, subsequent courtship encounters. We predicted that we could identify behavioral functions for these loci by testing mutations in the genes for effects on male courtship behavior.

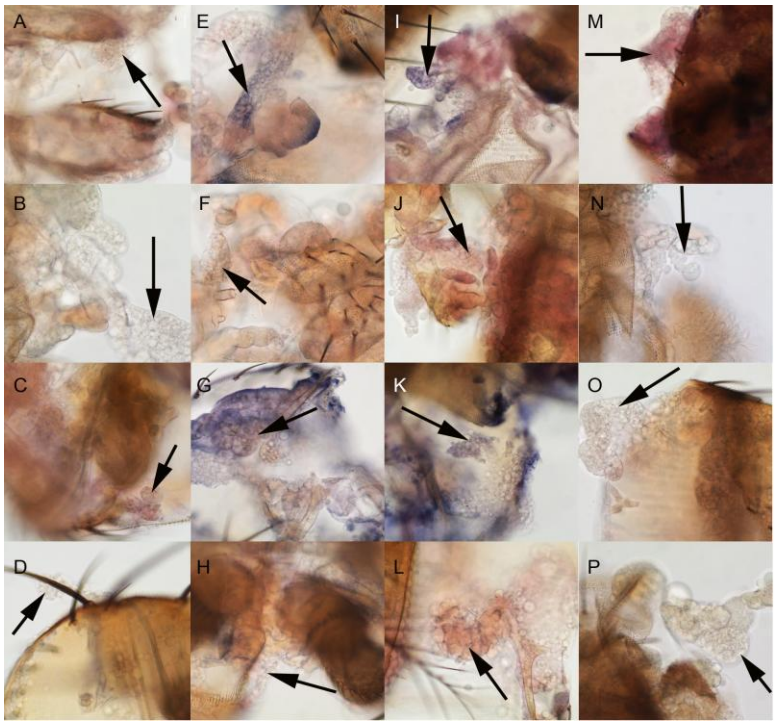


Figure 7. Courtship-responsive genes *CG10621, sug,* and *cwo* are expressed in male adipose tissue. Antisense (A,C,E,G,I,K,M,O) or sense (B,D,F,H,J,L,N,P) RNA probes were designed to cDNA clones for *CG9377* (A-D), *CG10621* (E-H), *sug* (I-L), *cwo* (M-N), and *egh* (O-P). *In situ* hybridization to whole-mounted male *CS* tissue shows candidate gene expression in the fat body tissue (arrows) on abdominal (A,B,E,F,I,J,M-P) or head (C,D,G,H,K,L) cuticle.

Therefore, we tested P-element insertions in courtship-responsive genes for

effects on male courtship activity (measured as the courtship index, CI). Males with either of 2 independent insertions in *egh* (*egh*^{EP804} and *egh*^{EY03917}) performed all of the standard courtship behaviors but had significantly reduced CI values compared to genetically similar controls (Figure 8) (two-tailed *t*-test, $p<0.001$). Therefore, reduced *egh* expression led to an overall reduction in time spent courting a female.

Reintroduction of a genomic copy of *egh* in the *egh*^{EY03917} background restored courtship activity to wild-type levels (Figure 9) verifying that the courtship phenotype is due to disruption of the *egh* locus. We selectively reduced *egh* in the adult nervous

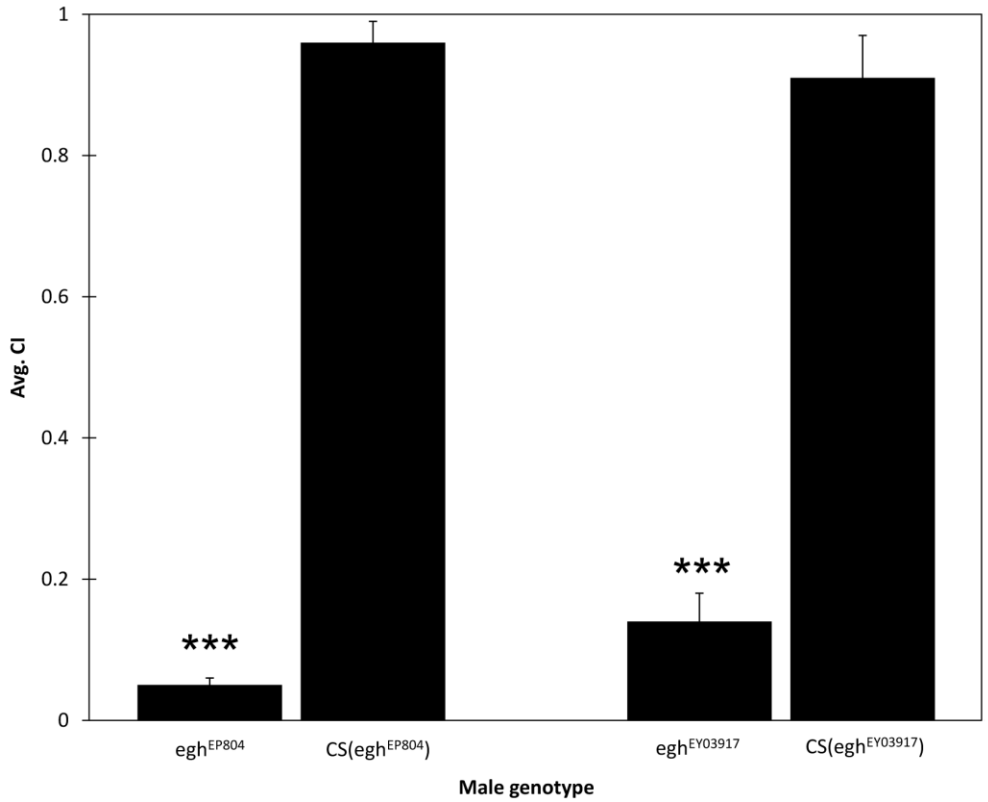


Figure 8. *egh* is required for robust male courtship behavior. Under red light, males with either X-linked *egh* insertion (*egh*^{EP804} or *egh*^{EY03917}) show significant (***$p<0.001$) decreases in CI values compared to control males in a similar genetic background (CS(*egh*^{EP804}) or CS(*egh*^{EY03917})) under similar conditions. Error bars reflect the SEM. N=10 for each genotype.

system with *UAS-egh*-RNAi under the control of *UAS-tubulin-Gal80^{ts}* and neural-expressed *elav^{c155}-Gal4.* This adult-specific decrease in *egh* resulted in significantly reduced CI values for experimental males at the restrictive temperature (29^{o}C) compared to all controls (Figure 10).

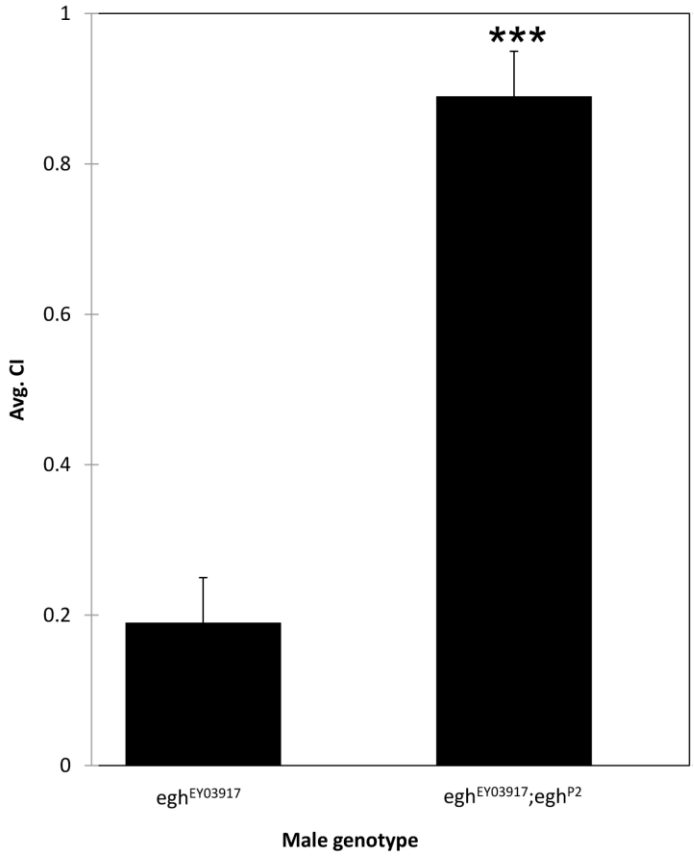


Figure 9. *egh* expression rescues male courtship behavior. Restoring *egh* expression in *egh*-expressing cells (*eghP2*) in the *egh^{EY03917}* mutant background significantly (***p<0.001) rescued the courtship defect in *egh^{EY03917}* mutant males. N=10 for both genotypes.

Larval *egh* expression is required in *ap*-expressing ventral nerve cord (VNC) neurons for the female Sex-peptide response during adulthood (Soller *et al.* 2006). Though Soller *et al.* (2006) attributed modulation of the Sex-peptide response to

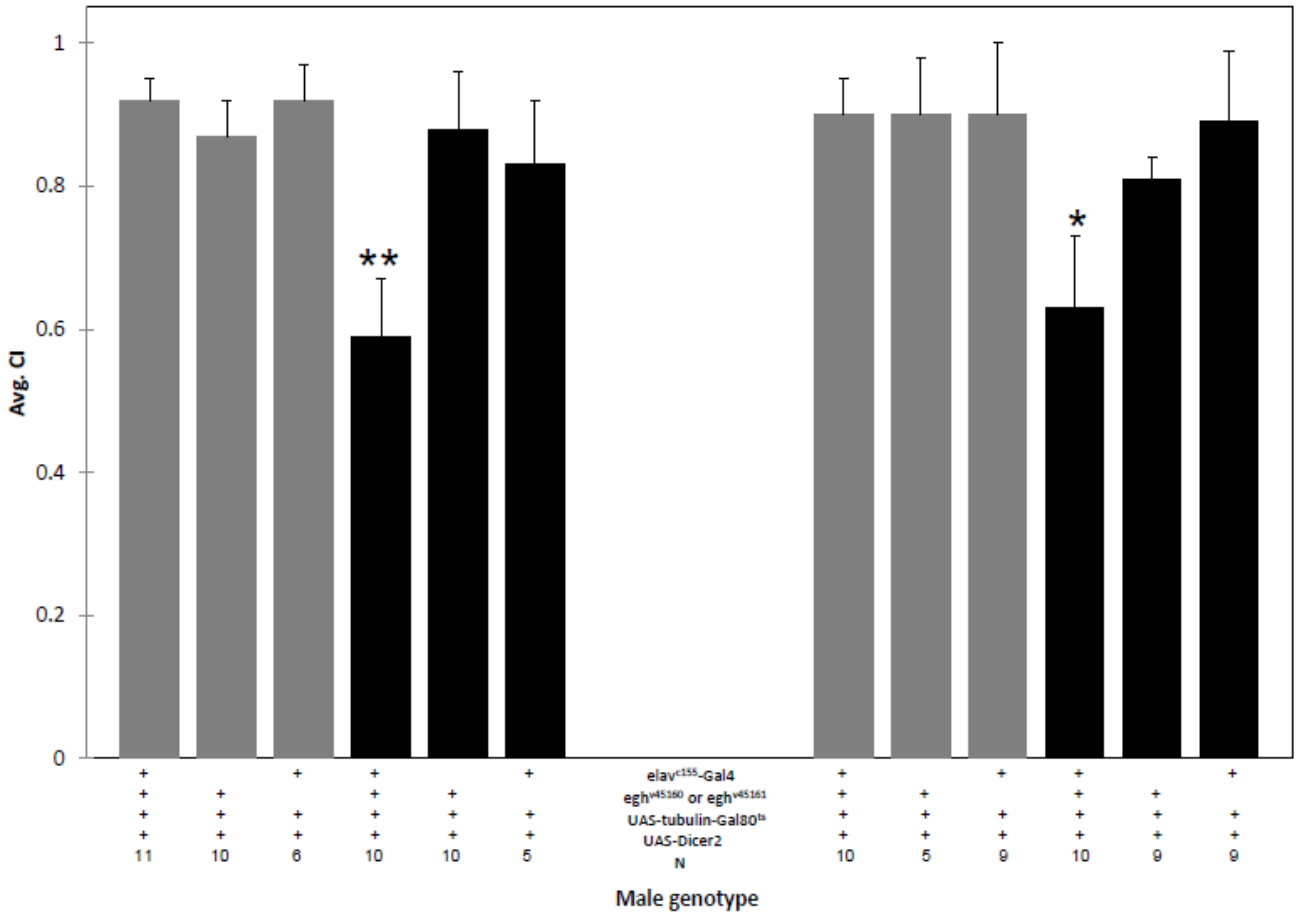


Figure 10. Male courtship requires *egh* expression in the adult nervous system. Expressing *UAS-egh*-RNAi alleles, egh^{v45160} or egh^{v45161}, in the adult nervous system using $elav^{c155}$*-Gal4*, *UAS-Dicer-2*, and *UAS-tubulin-Gal80*^{ts}, at the restrictive temperature (29°C, black bars) significantly (**p<0.01;*p<0.05) reduced male courtship activity compared to controls lacking $elav^{c155}$*-Gal4* or *UAS-egh*-RNAi compared to males at the permissive temperature (20°C, gray bars).

developmental expression of *egh*, *ap^{md544}-Gal4* expresses in the male and female adult nervous system (Figure 11). Therefore, we asked whether this same circuit functioned in male reproductive behavior. Expressing *egh* (via *UAS-eghHA*) under control of *ap^{md544}-Gal4* in *egh^{EY03917}* mutant males was sufficient to restore male courtship behavior (Figure 12), indicating that Ap neurons function to modulate reproductive behaviors in both sexes. We expressed *egh*-RNAi via *ap^{md544}-Gal4* to specifically reduce *egh* expression in adult males (Figure 13) and this targeted *egh* reduction resulted in decreased courtship activity. This indicates that *egh* is needed in Ap neurons during adulthood for proper courtship behavior.

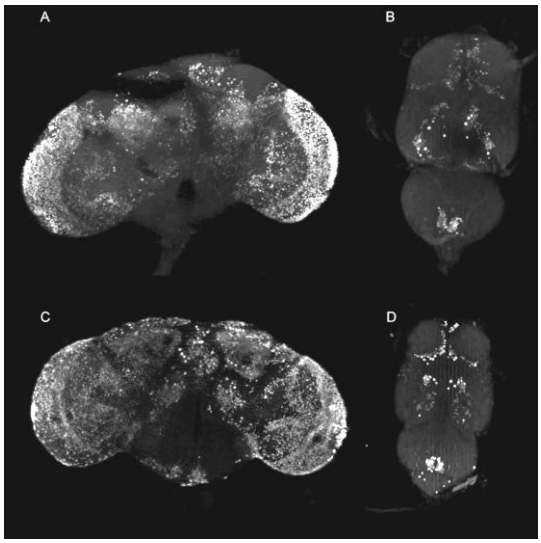


Figure 11. *ap^{md544}-Gal4* drives expression of GFP in the adult nervous system. Using *ap^{md544}-Gal4* to drive expression of GFP reveals *ap^{md544}-Gal4* activity in the adult brain (A, C) and VNC (B, D) of males (A, B) and females (C, D).

Discussion

Courtship interactions change male gene expression profiles

Drosophila perform stereotypical sex-specific courtship behaviors that are

influenced by genetics, including the somatic sex-determination pathway, and environmental cues, including social interactions. Previous studies have shown that courtship causes rapid (within 5 min) changes in whole-male transcript abundance (Carney 2007). These gene expression patterns may be altered directly or indirectly as a

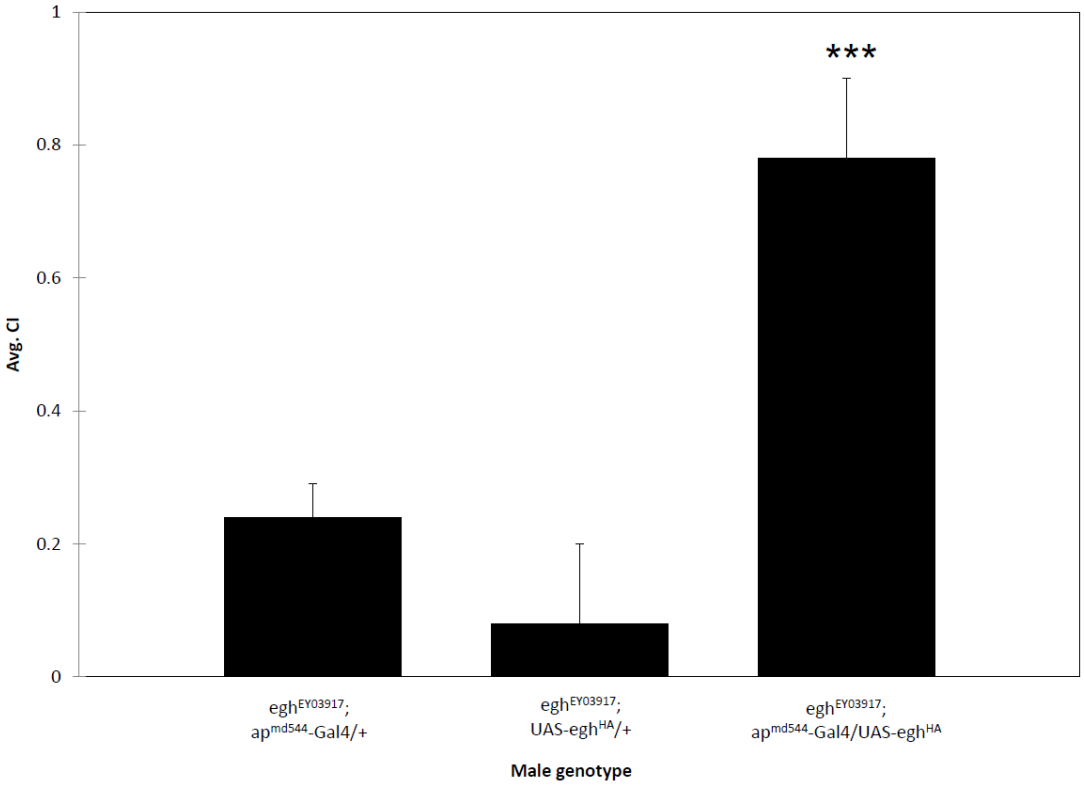


Figure 12. *egh* expression in *ap*-expressing neurons restores male courtship behavior. Narrowing *egh* expression to *ap* neurons by expressing UAS-*eghHA* under the control of ap^{md544}-*Gal4* in the $egh^{EY03917}$ background significantly (***p<0.001) restored male courtship activity compared to control $egh^{EY03917}$males lacking either component of the Gal4/UAS system. Ten males of each genotype were tested.

consequence of courtship. In this study we focused on male head tissue, showing that expression profiles of 35 (16 up-regulated and 19 down-regulated) genes differ after 20 min of courtship. Similarly to genes identified in array studies on songbirds and honey

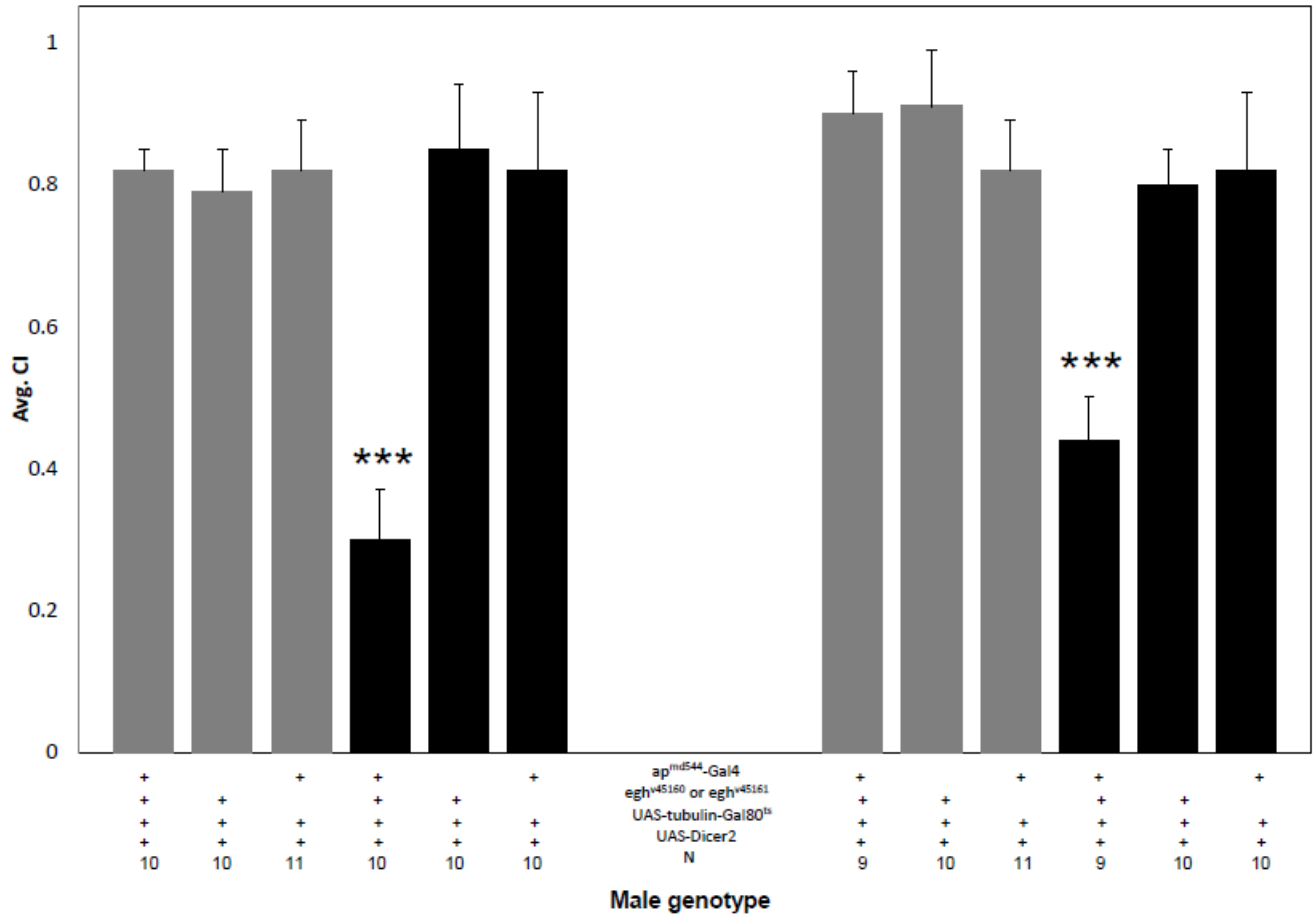


Figure 13. Adult expression of *egh* in *ap*-expressing neurons is necessary for robust courtship behavior. Expressing the *UAS-egh*-RNAi allele, egh^{v45160} or egh^{v45161}, in a subset of neurons during adulthood using ap^{md544}*-Gal4*, *UAS-Dicer-2*, and $UAS\text{-}tubulin\text{-}Gal80^{ts}$, under the restrictive (29°C, black bars) temperature significantly (***$p<0.001$) reduced male courtship activity compared to controls lacking the Gal4 or *UAS-egh*-RNAi component or compared to males at the permissive temperature (20°C, gray bars).

bees responding to behavioral cues (Grozinger *et al.* 2003; Whitfield *et al.* 2003; Wada *et al.* 2006; Whitfield *et al.* 2006; Sen Sarma *et al.* 2009), Drosophila courtship-responsive genes have known or predicted functions in a variety of important cellular processes, including transcription and neuronal development and signaling (Tables 7 and 8).

We predicted that some courtship-responsive loci would function as downstream targets of the somatic sex-determination pathway that regulates male courtship behavior. Indeed, 3 courtship-responsive genes are regulated by this pathway. *fit* is regulated by *transformer* (*tra*), *CG9377* is downstream of *fruitless (fru)*, and *CG9837* is regulated by *doublesex (dsx)* (Goldman and Arbeitman 2007).

Due to several differences in experimental design between our earlier studies and the study described here (different time points and tissues examined, use of a newer version of the Drosophila genome array, use of different data analysis methods), there was little overlap between the courtship-responsive gene lists. However, one gene, *fit*, was up regulated after 5 min and 20 min of courtship. *CG2217* was up regulated in whole males after 5 min of courtship, but after 20 min of courtship it was down regulated in the head. The decreased expression of *CG2217* may be specific to the male head, while its increase during 5 min of courtship occurs elsewhere in the body.

Gene expression in the male brain

Since brain gene expression has a clear function in behavior, we expected that

some courtship-responsive genes would be expressed in the brain. Therefore, we examined brain tissue for expression of a subset of the courtship-responsive genes. *CG9377* and *egh*, which increase in abundance due to courtship, were expressed in the male brain but not detected in adipose tissue (Figures 6 and 7). Two of the down-regulated courtship-responsive genes are known to function in behavior and are expressed in the brain. *cacophony* encodes a calcium voltage-gated channel needed for proper pulse frequency and amplitude during courtship song production (Billeter *et al.* 2002; reviewed in Greenspan and Ferveur 2000); *Shaker* (*Sh*) encodes a potassium channel that functions in olfactory memory and learning (reviewed in Greenspan and Ferveur 2000).

Several courtship-responsive genes (*Drop, egh, hairy, lola,* and *Sh*) regulate nervous system development and function (Giniger *et al.* 1994; Heng *et al.* 2003; Zhong and Wu 2004; Fan *et al.* 2005; Ueda and Wu 2006; Urbach *et al.* 2006) and could act to modulate adult neural signaling and courtship. Changes in brain gene expression patterns due to courtship interactions are likely a result of signaling pathways, including G-protein couple receptor signaling, functioning within the brain to mediate the perception and integration of sensory cues. Such signaling pathways may coordinate motor output pathways necessary for courtship and relay information to the brain to establish a male brain that is more readily perceptive to courtship cues than a naïve male brain.

Gene expression in male adipose tissue

Signals mediating courtship cues are not likely restricted to the brain, however. Adipose tissue, or the fat body, surrounding the brain and in the thoracic and abdominal cavities is a secretory tissue (reviewed in Schlegel and Stainier 2007) that could influence neuronal signaling or transmit signals to other reproductively important tissues. Indeed, there is a growing body of evidence that fat body-expressed genes modulate reproductive behaviors (reviewed in Dauwalder 2008).

fit and *Lsp2* are expressed in the female and male fat body (Benes *et al.* 1990; Fujii and Amrein 2002), and *in situ* hybridization confirmed the fat body expression of 3 additional courtship-responsive genes (*CG10621*,*cwo*, and *sug*) (Figure 7). *cwo* is also expressed in the male brain, but we could not detect *CG10621* or *sug* transcripts in the male brain. Many courtship-responsive genes are enriched in head tissue, including the fat body but not including the brain (Figures 6 and 7; Table 6). This suggests that the circuitry responding to and governing courtship behavior likely is modulated by both neuronal and non-neuronal signals. The response to courtship involves complex and specific changes that may mediate various downstream effects including neural plasticity.

egghead and courtship behavior

To determine if mutations in courtship-responsive genes affect courtship, we measured CI values in P-element insertion mutants. Our analysis showed that a specific courtship-responsive locus, *egh*, is needed in a particular subset of neurons during

adulthood for robust male courtship behavior. *egh* is up regulated 20 min after courtship, and mutations in *egh* disrupt courtship activity (Figures 8, 10, and 13); restoring genomic *egh* expression rescues this phenotype (Figure 9). We expressed RNAi alleles that target *egh* via $elav^{c155}$*-Gal4* under control of the temperature-sensitive Gal80 and determined that *egh* expression is required in adult male neurons for proper courtship behavior (Figure 10).

egh encodes a β1,4-mannosyltransferase which regulates glycosphingolipid biosynthesis (Wandall *et al.* 2003) and affects Drosophila neural development and behavior. This mannosyltransferase is needed in optic lobe development (Fan *et al.* 2005) and is required for female Sex-peptide response (Soller *et al.* 2006). Males transfer sperm, accessory gland proteins, and Sex-peptide to females during mating. Sex-peptide causes post-mating responses in females, including increased ovulation and egg laying and decreased receptivity (Wolfner 2009). Soller *et al.* (2006) showed that *egh* is needed in *ap*-expressing ventral cord neurons of female larvae for the Sex-peptide induced post-mating response in adulthood. Since ap^{md544}*-Gal4* is expressed in the adult nervous system of both sexes (Figure 11) we examined whether this same neural circuit functioned in males to regulate courtship behavior. In $egh^{EY03917}$ mutant males, *egh* expression in Ap neurons was sufficient to rescue the courtship defect (Figure 12). We decreased *egh* expression (via RNAi) in *ap*-expressing neurons during adulthood and found that this adult-specific *egh* reduction resulted in decreased courtship (Figure 13). Similarly to males with reduced adult *egh* expression, *ap* mutant males have decreased levels of male-to-female courtship (Ringo *et al.* 1992). Ap is a transcription factor that

regulates developmental as well as post-developmental neural gene expression (Benveniste *et al.* 1998). Given the similarity between the *ap* and *egh* mutant phenotypes and the requirement for *egh* expression in *ap* neurons for male courtship, it is possible that *ap* regulates *egh* expression.

Male and female Drosophila perform sex-specific behaviors. At the heart of these differences lies the *fru* circuit. Sex-specific differences in behaviors may be due to dimorphisms in neural architecture, including the number or morphology of neurons, such as those present in the *fruP1* circuit that modulates male courtship behavior (Kimura *et al.* 2005; Stockinger *et al.* 2005; Rideout *et al.* 2007; Clyne and Miesenböck 2008; Datta *et al.* 2008). On the other hand, the same circuit could be co-opted by each sex for different behaviors. We hypothesize this is the case for the *egh* circuit. *egh* is required in Ap neurons in both males and females but modulates sex-specific reproductive behaviors. This may occur because of changes in neural physiology resulting from the perception of sex-specific cues that trigger different signaling cascades between the sexes. However, it is possible that different subsets of Ap neurons regulate sex-specific behavior. The *egh* circuit important for male behavior does not appear to rely directly upon *fru* neurons since expressing *egh* in *fru* neurons did not rescue the behavioral defects observed in *egh* mutant males. Therefore, *egh* neurons may interact indirectly with *fru* neurons to modulate Drosophila reproductive behaviors.

Our study strengthens the growing body of work demonstrating that animals respond to social interactions by altering transcript abundance. By investigating the function of these courtship-responsive loci, we can clarify the relationship between

genetics and the intracellular processes governing behavior. In Drosophila, courtship-responsive loci include known sex-determination hierarchy target genes, and further characterization of courtship-responsive genes will likely reveal more genes functioning within this pathway.

CHAPTER IV

*fit*TING IT ALL TOGETHER: HOW THE COURTSHIP- AND MATING-RESPONSIVE GENE *fit* AFFECTS MALE *Drosophila melanogaster* COURTSHIP BEHAVIOR

Introduction

Reproductive success requires that an organism be able to properly perceive and interpret sensory cues in order to discern suitable from non-suitable mates. The ability to attract proper mates is also key to reproductive success. We utilize the genetically tractable *Drosophila melanogaster* to further understand these processes.

Drosophila melanogaster perform stereotypical sex-specific behaviors (reviewed in Greenspan 1995; Greenspan and Ferveur 2000) that are modulated by genetics (reviewed in Tompkins 1984; Billeter *et al.* 2002) and social interactions (Siegel and Hall 1979; Dukas and Mooers 2003; Siwicki and Ladewski 2003; Ellis and Carney 2009; reviewed in Ewing 1983; Greenspan and Ferveur 2000; Mehren *et al.* 2004) and which are governed largely by the somatic sex-determination hierarchy (reviewed in Cline 2005; Shirangi and McKeown 2007). This genetic pathway also regulates sexually dimorphic development (Finley *et al.* 1997; Demir and Dickson 2005; Kimura *et al.* 2005; Manoli *et al.* 2005; Stockinger *et al.* 2005; Billeter *et al.* 2006; Rideout *et al.* 2007; Sanders and Arbeitman 2008; Mellert *et al.* 2010; Rideout *et al.* 2010). Few of the known transcriptional targets of this pathway (Burtis *et al.* 1991; Cann *et al.* 2000; Kopp *et al.* 2000; Dauwalder *et al.* 2002; Fujii and Amrein 2002; Drapeau *et al.* 2003;

Arbeitman *et al.* 2004; Goldman and Arbeitman 2007; Lazareva *et al.* 2007; Fujii *et al.* 2008; Dalton *et al.* 2009) have known roles in nervous system function or behavior.

One sex-determination target gene of interest is *female-specific independent of transformer* (*fit*). Although named for its higher level of expression in females compared to males and the *Sex-lethal* (*Sxl*)-dependent regulation of its expression (Fujii and Amrein 2002), its expression is also dependent on *transformer* (*tra*) (Goldman and Arbeitman 2007). *fit* is enriched in adipose tissue known as the fat body (Fujii and Amrein 20020), a secretory tissue (reviewed in Schlegel and Stainier 2007) that has recently been implicated in modulating behavior (Dauwalder *et al.* 2002; Fujii and Amrein 2002; Lazareva *et al.* 2007; Fujii *et al.* 2008; reviewed in Dauwalder 2008). Our previous work has shown that *fit* is increased in courting males compared to control males (Ellis and Carney 2009). Here we provide the first evidence of *fit's* role in behavior. Examining *fit* knock-out or *UAS-fit* strains as well as available *UAS-fit*-RNAi alleles revealed *fit's* role in the adult fat body for repressing male-male courtship. Mutant *fit* males court control males and are also courted by control males.

Materials and Methods

In situ hybridization

Antisense and sense RNA probes were designed to *fit* using the cDNA clone RH40291 and the digoxigenin (DIG)-labeling kit following the manufacturer's protocol (Roche, Nutley, NJ, USA). Probes were hydrolyzed into 200 bp fragments as previously described (Arbeitman *et al.* 2004). We verified the increased expression of *fit* in male

head tissue after courtship or after courtship followed by mating. One group of virgin *CS* males courted a cauterized female for 20 min, while a control group of virgin *CS* males were not given a female courtship object. We also tested *fit* expression in virgin *CS* males 2 hrs after mating compared to control males that did not mate with a female. After treatment, males were cryo-sectioned in OCT compound and i*n situ* hybridization was performed on the sections (Dauwalder *et al.* 2002). We qualitatively assessed *fit* expression in adipose tissue lining the head, thoracic or abdominal cuticle from non-existent (-) to highly expressed (+++).

Generating the *fit* knock-out mutant

Following Maggert *et al.* (2008) we designed primers to sequences 3968bp upstream and 4219bp downstream of the *fit* locus. We sequentially cloned in these flanking sequences into the pCR-BluntII-TOPO cloning vector (Invitrogen, Carlsbad, CA, USA) for subsequent cloning into the pW25.2 vector. We cloned 4000 bp upstream of the *fit* locus between the BsiWI and AscI restrictions sites and 4200 bp downstream of the *fit* locus between the NheI and MluI sites. The construct was injected into *w* embryos by Genetic Services (Cambridge, MA) following standard protocols. Transgenic lines were crossed to FLPase, I-SceI flies for recombination between the donor and endogenous chromosomes. Two independent strains (fit^{NNN1} and fit^{T15}) with properly targeted recombination events, as determined both phenotypically and by PCR amplification, were then crossed to CreRec flies for removal of the w^+ marker. We sequenced across the *fit* locus to verify the deletion of *fit* in both strains.

pUASp-fit cloning

We cloned the *fit* cDNA sequence from the RH40291 downstream of the UAS sequence in the *pUASp* vector at the NotI and BamHI sites. The construct was sequenced and then injected into *w* embryos by Duke University Model System Genomics (Durham, NC).

Immunostaining

A peptide antibody was generated against the Fit peptide sequence (PHSVNWPCDVGHFPE) downstream of the predicted signal sequence. The peptide was injected into rabbits by Sigma Genosys following their standard protocol. The serum from the final bleed was affinity purified. Dissected female spermathecae and the surrounding fat from homozygous knock-out and heterozygous control animals were stained with a 1:1 concentration of anti-Fit antiserum and 1:1500 of Alexa Fluor goat anti-rabbit 594 secondary antibody (Molecular Probes) following Boltz *et al.* (2007). A Zeiss Axio Imager Z1 fluorescent microscope was used for imaging.

Courtship behavior analysis

Flies were maintained on a 12-hr light/dark cycle at 25^{o}C. All flies were collected as virgins within 2 hrs of lights on. The two deletion strains (fit^{NNN1} or fit^{T15}) have a balancer chromosome floating in the stock to generate the heterozygous control genotypes. fit^{NNN1} and fit^{T15} were crossed to each other to generate transheterozygous mutants for behavioral analysis. Since knock-out flies are white eyed we conducted all

behavioral assays under dim, red light to offset the effects vision has on courtship.

Male-female courtship behavior

fit mutant or heterozygous males were aged individually for 4 to 5 days and *CS* virgin females were aged collectively for 3 to 5 days at 25^{o}C. Behavioral assays took place at 22^{o}C in courtship chambers (1cm diameter). On day 5, a male was aspirated into a chamber and then a female was aspirated in. The pair was video recorded for 10 min and the courtship index (CI; ratio of time spent in courtship compared to the total observation window) was calculated. CI values were arcsine transformed for statistical analyses (ANOVA and Tukey's).

Male-male courtship behavior

Virgin *fit* knock-out, *fit*/+, or *CS* males that served as courters were aged individually and those that were the courtship objects were aged collectively for 4 to 5 days at 25^{o}C. Courtship object males were decapitated, to prevent courtship rejection behaviors, 10 min before testing. The CI for various pairings of control and mutant males was measured by aspirating a courter male into the courtship chamber, followed by the decapitated courtship object, at 22^{o}C, and the pairs were video recorded for 10 min.

Using two *UAS-fit*-RNAi alleles (*fit*^{v14433} or *fit*^{v14434}) from the Vienna Drosophila RNAi Center (Dietzl *et al.* 2007) we were able to reduce *fit* expression in the fat body

(with *3.1Lsp(2)-Gal4* (Lazareva *et al.* 2007) or *Cg-Gal4* (Hennig *et al.* 2006)) or nervous system (with *elav^{c155}-Gal4* (Lin and Goodman 1994)). Crosses were maintained at 25^{o}C and courtship assays were performed, as previously mentioned, at 29^{o}C. *In situ* hybridization confirmed reduced levels of *fit*.

For adult-specific reduction of *fit,* we introduced the temperature-sensitive *UAS-tubulin-Gal80^{ts}* (McGuire *et al.* 2004). Crosses were maintained at the permissive 20^{o}C and virgin males were collected and housed at either 20^{o}C or 29^{o}C for testing at their respective temperatures.

Male courtship preference

To determine if *fit* males preferred to court *CS* males or females, we collected virgin *fit* mutant or control males and aged them individually for 4 to 5 days. Virgin *CS* males or females were collected and aged collectively for 5 days. *CS* flies were decapitated 2-10 min before behavioral assays. The decapitated courtship objects were aspirated into the courtship chamber and then the *fit* mutant or control male was aspirated into the chamber and the trio was recorded for 10 min. CI values for courtship towards male or female courtship objects were measured.

Fertility assays

A *fit* mutant or control male was paired with a virgin *CS* female, or a *CS* male was paired with a *fit* mutant or control female, in a vial with food to measure fecundity (number of adult offspring sired). The pairs were transferred to a new vial for 5

consecutive days and for each vial the number of eggs in each vial was counted. After 18 days, the number of adults was also counted and the ratio of adult offspring to eggs laid was calculated. ANOVA and Tukey's analyses determined significance ($p<0.05$).

Results

Courtship or mating up regulate *fit* expression in adipose tissue

Previous work in our lab has shown that *fit* is up regulated after courtship in whole bodies or heads (Carney 2007; Ellis and Carney 2009) and 2 hrs after mating (L. L. Ellis & G. E. Carney, unpublished results). *fit* is expressed the fat body of males and females (Fujii and Amrein 2002). *In situ* hybridization revealed that *fit* expression increased in the adipose tissue surrounding the male brain after courtship (Figure 14) or courtship followed by mating.

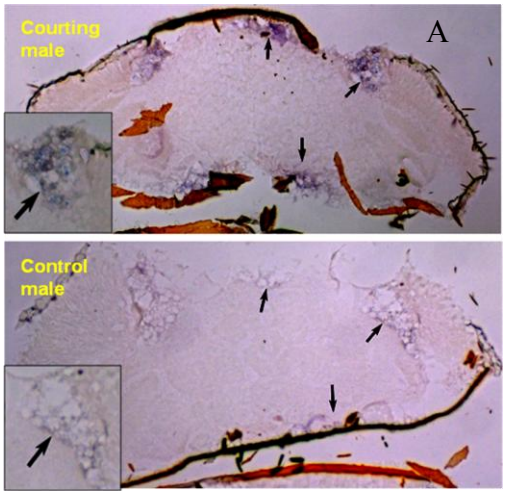
Fit expression in the fat body

Using anti-Fit antiserum we show that Fit localized to the adipose tissue in control females (fit^{NNN1}/+ *or* fit^{T15}/+) but was absent in knock-out females (fit^{NNN1}or fit^{T15}) (Figure 15).

Reduced *fit* expression results in increased male-male courtship

Since *fit* is responsive to courtship and mating encounters and is regulated by the somatic sex-determination hierarchy, we hypothesized that *fit* would likely function in reproductive behavior. To examine this, we tested *fit* deletion males for changes in

courtship activity (courtship index, CI), courtship latency, mating latency, mating duration, fertility and fecundity.



B

	- +	++	+++
Courting males	1	10	3
Mated males	4	13	3
Control males	25	2	1

Figure 14. Courting males show increased *fit* expression in the fat body. DIG-labeled *fit* RNA antisense and sense probes were made from the RH40291 cDNA clone. *In situ* hybridization was performed on cryo-sectioned male heads and confirmed that *fit* transcript levels are up regulated in the adipose tissue (arrows) of courting males (A. top) compared to control males (A. bottom), as seen by the (B) qualitative assessment of signal intensity in both treatment groups.

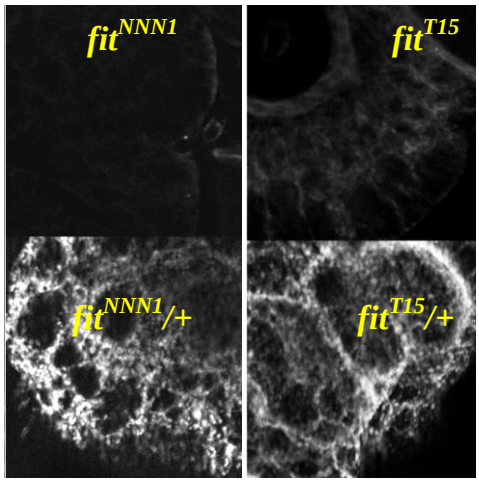


Figure 15. Fit is expressed in adipose tissue. Using anti-Fit antiserum, Fit is absent in knock-out mutants (fit^{NNN1} or fit^{T15}) but localizes to adipose tissue in (fit^{NNN1}/+ or fit^{T15}/+) females.

Deletion of *fit* did not affect mating latency, mating duration, fertility or fecundity. *fit* null and control males courted *CS* females at a similar level (Figure 16). Since mutations in some sex-determination genes cause male-male courtship, we tested whether or not *fit* mutant males courted males. Deletion of *fit* increased courtship towards a male of the same genotype, but *fit* heterozygous mutants did not court each other (Figure 17). The increased male-male courtship could be due to the inability of *fit* homozygotes to properly identify males as inappropriate courtship objects (referred to as the bisexual phenotype since *fit* mutant males also court females; see Figure 16) or because these mutants are eliciting courtship from other males (referred to as the elicitation phenotype). To address which of these, or if both, scenarios are the reason for this increased male-male courtship we tested various combinations of mutant and control male-male pairings. *fit*-null males court control (*fit/+* or *CS*) males but are also courted by control males (Figure 17). The increased courtship activity is not due to increased locomotion since mutants and controls showed similar locomotory activity (*t*-test; $p>0.05$). Since *fit* mutant males show no male-female courtship defects but show increased male-male courtship, we asked whether *fit* mutant males preferred to court *CS* males or *CS* females. *fit* mutant males courted females for significantly more time than they courted males (Figure 18), suggesting that *fit* males can detect female cues but cannot respond properly to male inhibitory cues.

Restoring *fit* expression by expressing *UAS-fit* with *actin-Gal4* reduces both the bisexual and elicitation phenotypes of male-male courtship indicating that these phenotypes are due to loss of *fit* (Figure 19).

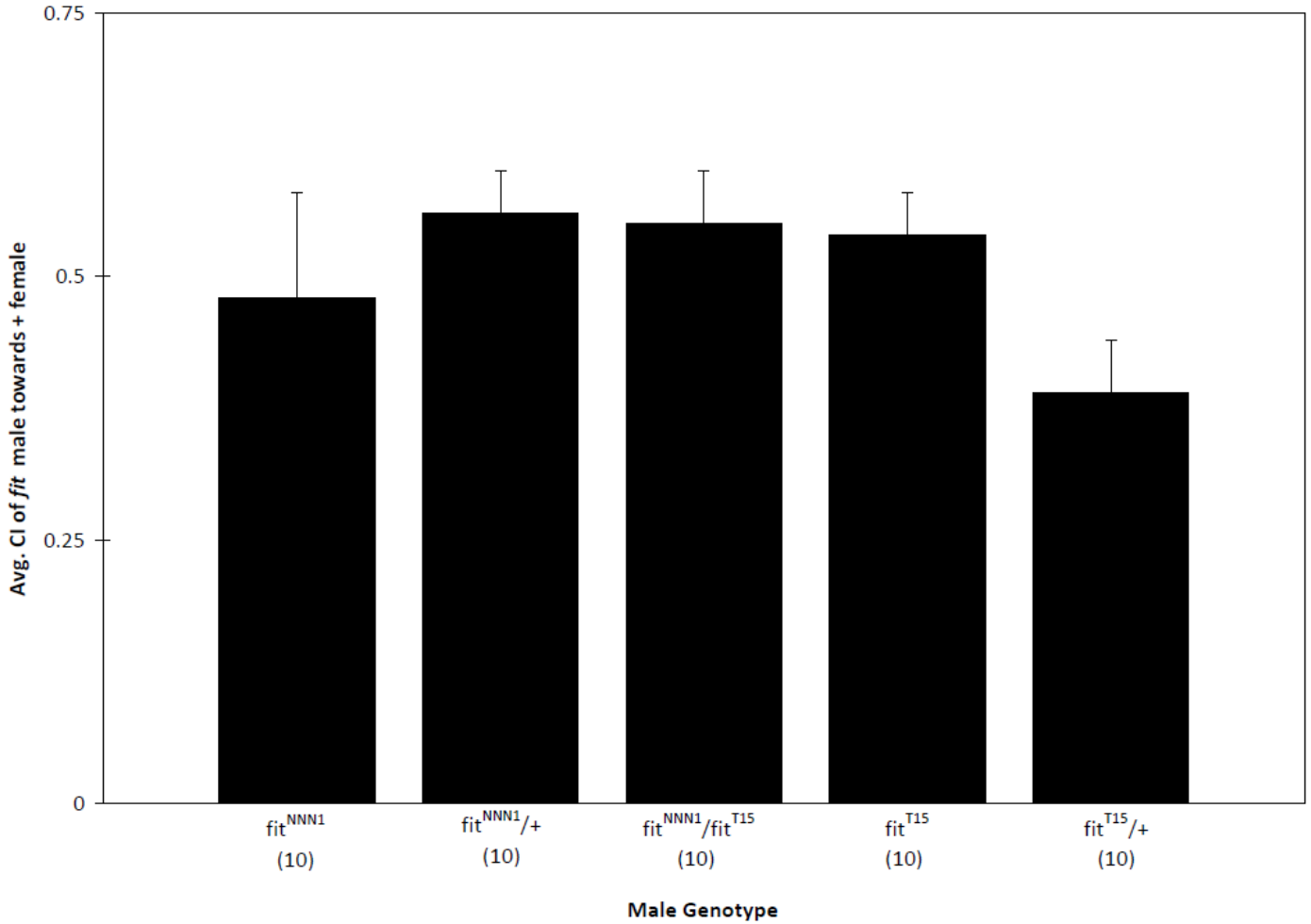


Figure 16. Deletion of *fit* does not affect male-female courtship activity. Homozygous knock-out or transheterozygous mutant males show similar courtship levels (as measured by CI values) compared to heterozygous controls.p>0.05 (N=sample size).

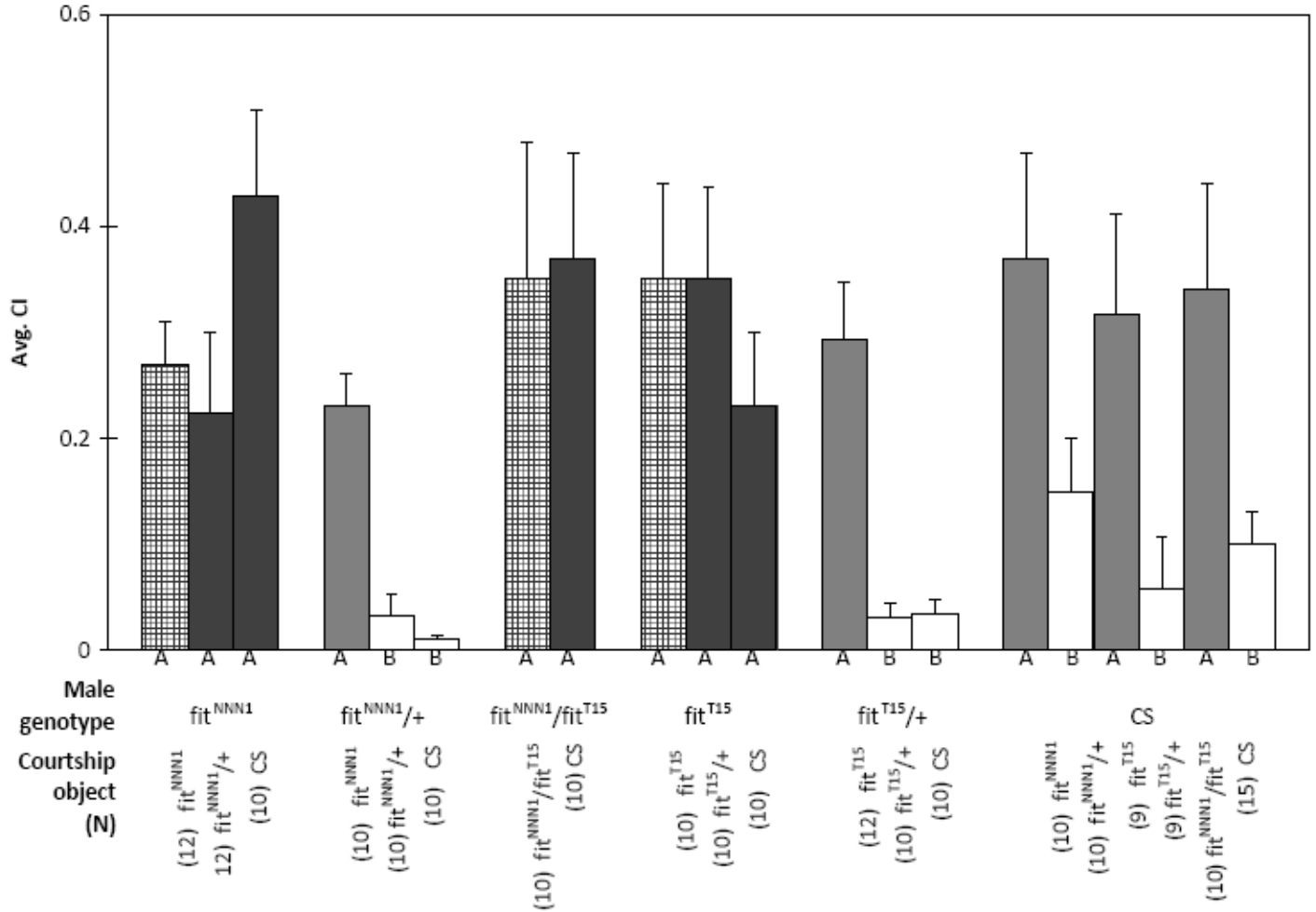


Figure 17. *fit* is necessary to repress male-male courtship. We tested various combinations of mutant and control males to determine if male-male courtship exists. Since *fit* males court *fit* males (checkered bars), we asked if they are unable to identify proper mates (dark gray bars) or if *fit* mutants elicit courtship (light gray bars) compared to controls (white bars). A & B denote statistical groups such that A is significantly different than B at $p<0.05$. (N=sample size).

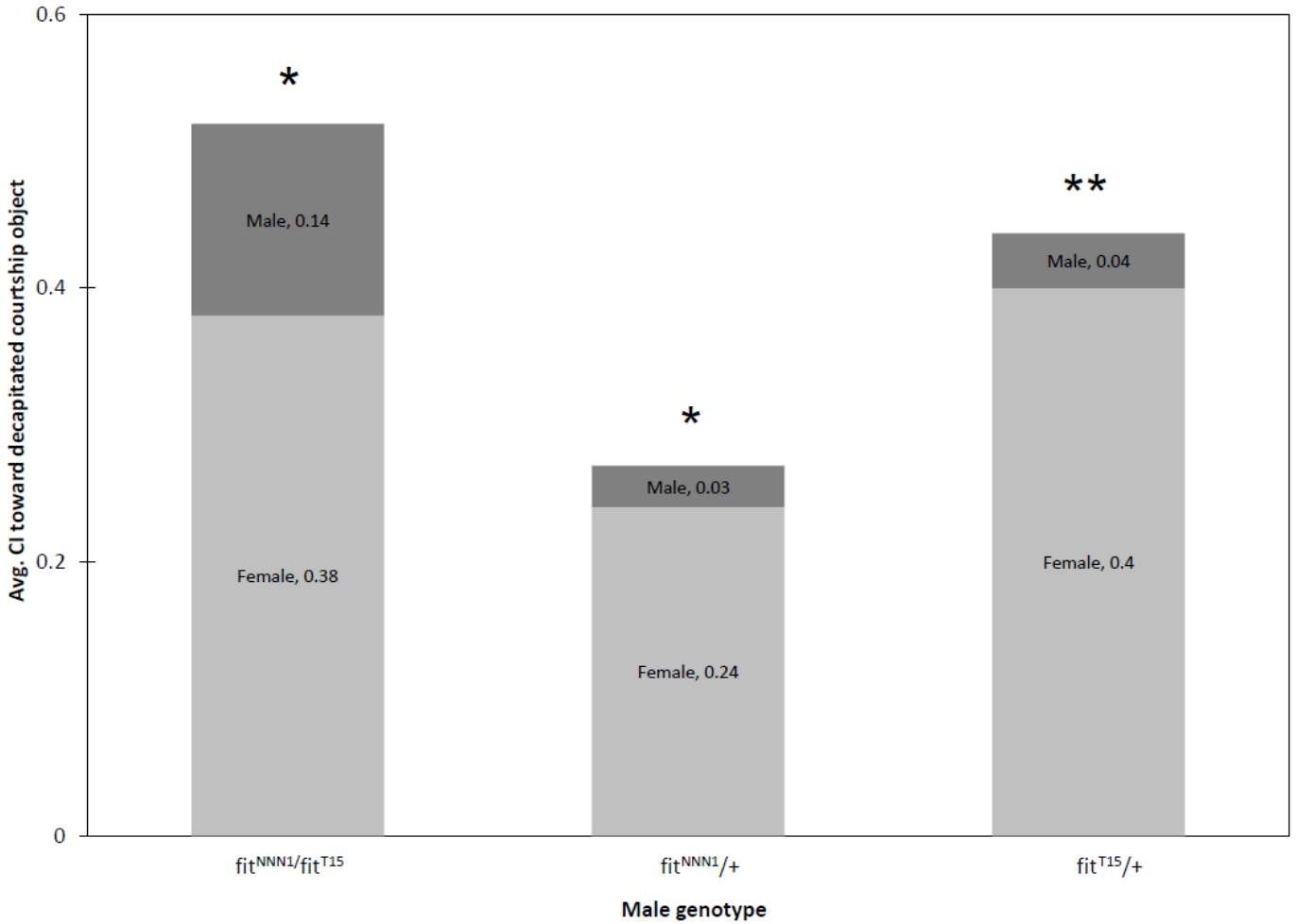


Figure 18. *fit* mutant males prefer female courtship objects. When given both courtship objects (a decapitated female and a decapitated male), fit^{NNN1}/fit^{T15} males, as well as control males (fit^{NNN1}/+ or fit^{T15}/+) court females (light gray bars) more often than males (dark gray bars). *p<0.05; **p<0.01 (N=sample size).

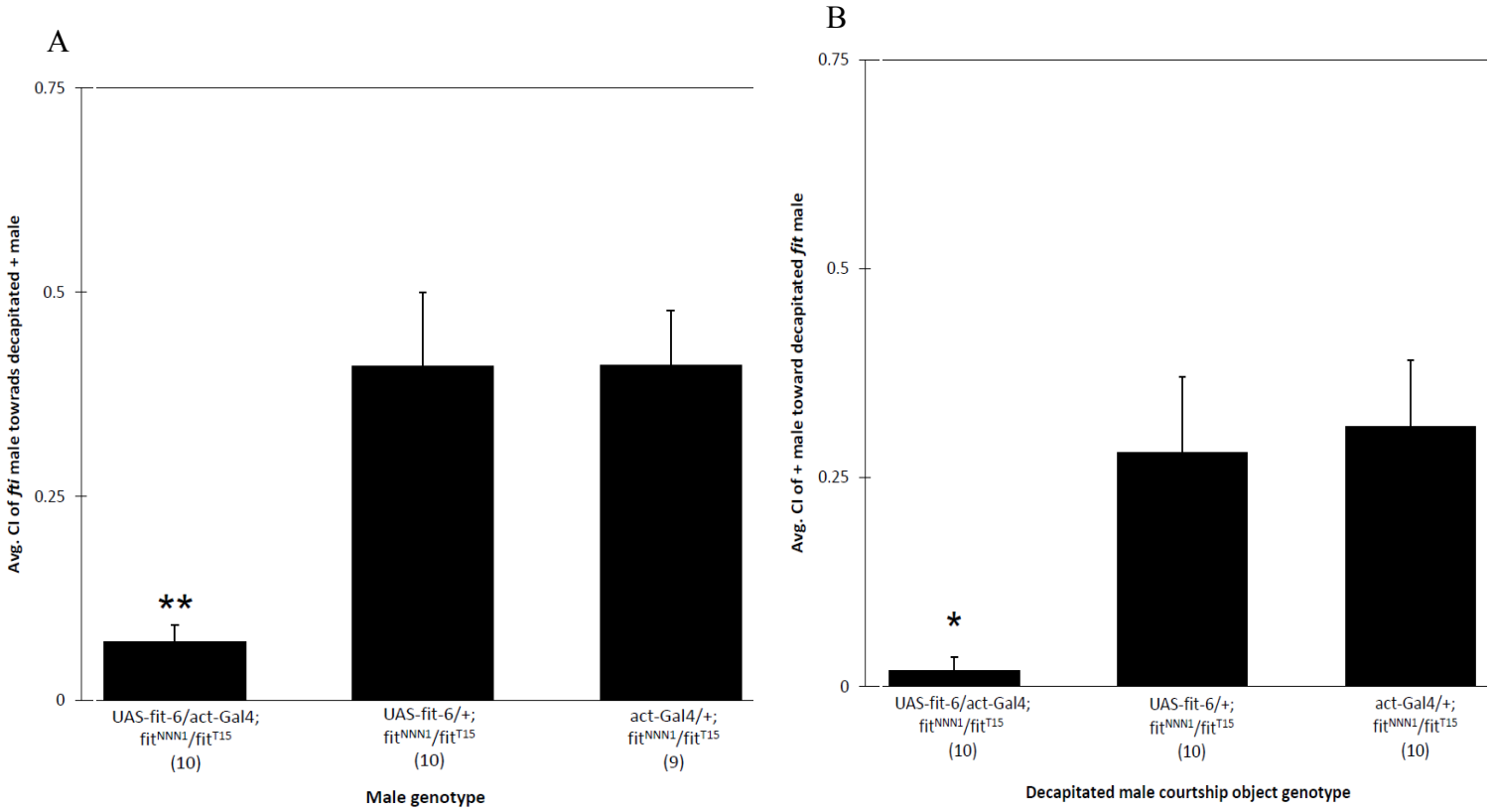


Figure 19. Expression of *fit* decreases male-male courtship. Over-expressing *fit* with *actin-Gal4* in the transheterozygous mutant background reduces the courtship activity of (A) *fit* males toward control males or (B) control males toward *fit* males (*p<0.05; **p<0.01). (N=sample size).

Since *fit* is expressed in adipose tissue, which modulates reproductive behaviors (Dauwalder *et al.* 2002; Fujii and Amrein 2002; Lazareva *et al.* 2007; Fujii *et al.* 2008; reviewed in Dauwalder 2008), we reduced *fit* expression in the fat body with *fit*-targeted RNAi (*fit*^{v14433} or *fit*^{v14434}) using either *3.1Lsp(2)-Gal4* (Lazareva *et al.* 2007) or *Cg-Gal4* (Hennig *et al.* 2006). Reduced *fit* expression in the fat body resulted in both the bisexual and elicitation male-male courtship phentoypes (Figure 20).

We asked if *fit's* role in repressing male-male courtship was due to developmental or adult-specific requirements. Combining *UAS-tubulin-Gal80*^{ts}, *UAS-fit-RNAi* and fat body drivers, we were able to decrease *fit* in the adult fat body. Our results show that *fit* expression was required in the adult fat body to repress male-male courtship (Figure 21).

We are currently testing whether or not *fit* expression in the fat body is sufficient to repress the bisexual and elicitation phenotypes. Also, we are examining if *fit* expression in the nervous system modulates male-male courtship.

Discussion

Identifying a courtship- and mating-responsive gene

The sex-specific courtship behaviors performed by Drosophila involve the perception and interpretation of sensory information. These processes are modulated by genetics and experience; therefore, we examined the gene expression changes in Drosophila males after courtship (for 5 min or 20 min) or 2 hrs after courtship culminating in copulation. We found that one gene, *fit*, is up regulated in each of the

A

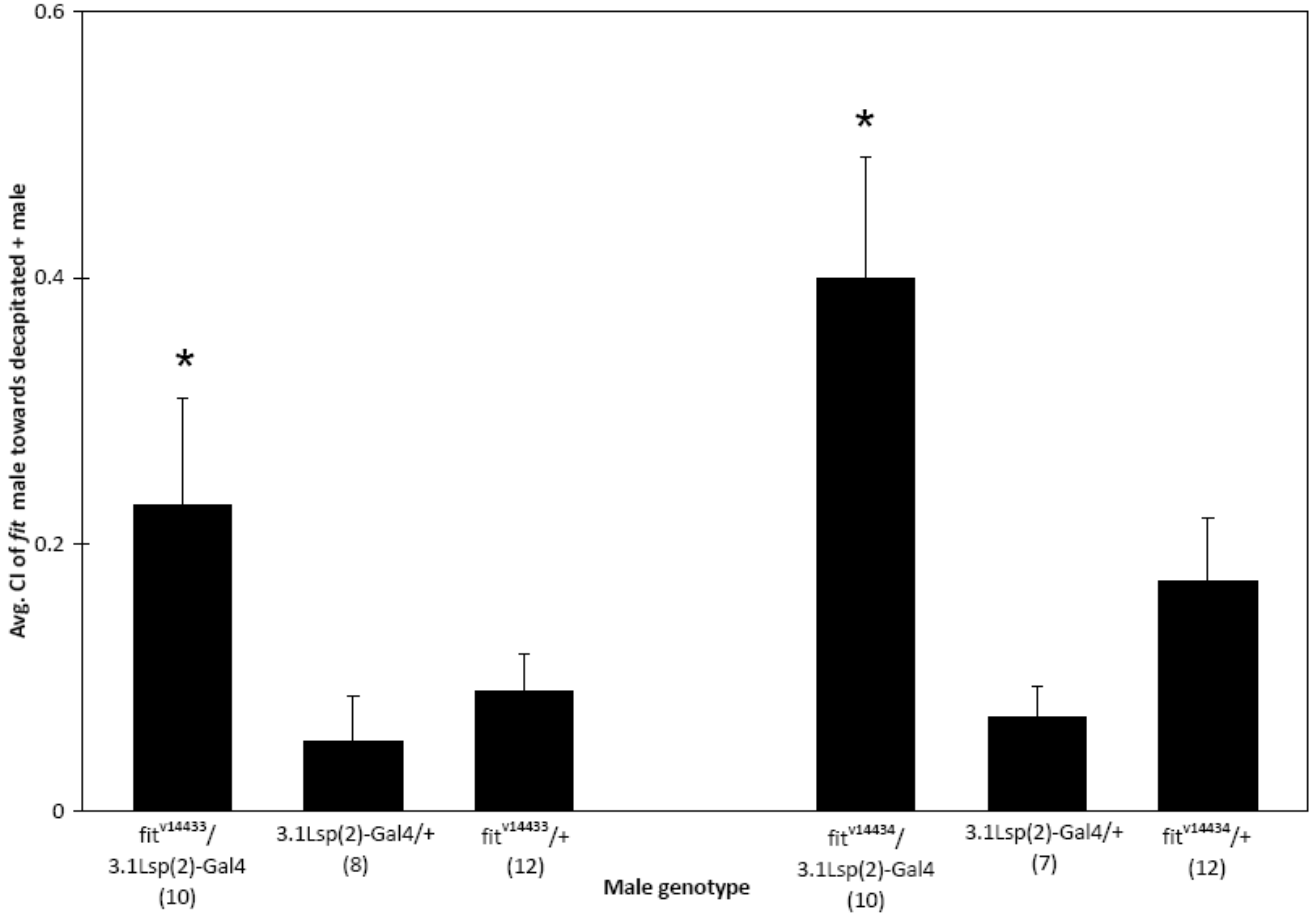


Figure 20. Reduced *fit* expression in the fat body results in male-male courtship. Expressing *UAS-fit*-RNAi (*fit*^{v14433} or *fit*^{v14434}) with 3.1Lsp(2)-Gal4 resulted in *fit* males that increased courtship towards *CS* males (A) and from *CS* males (B) ($p<0.05$). (N=sample size).

B

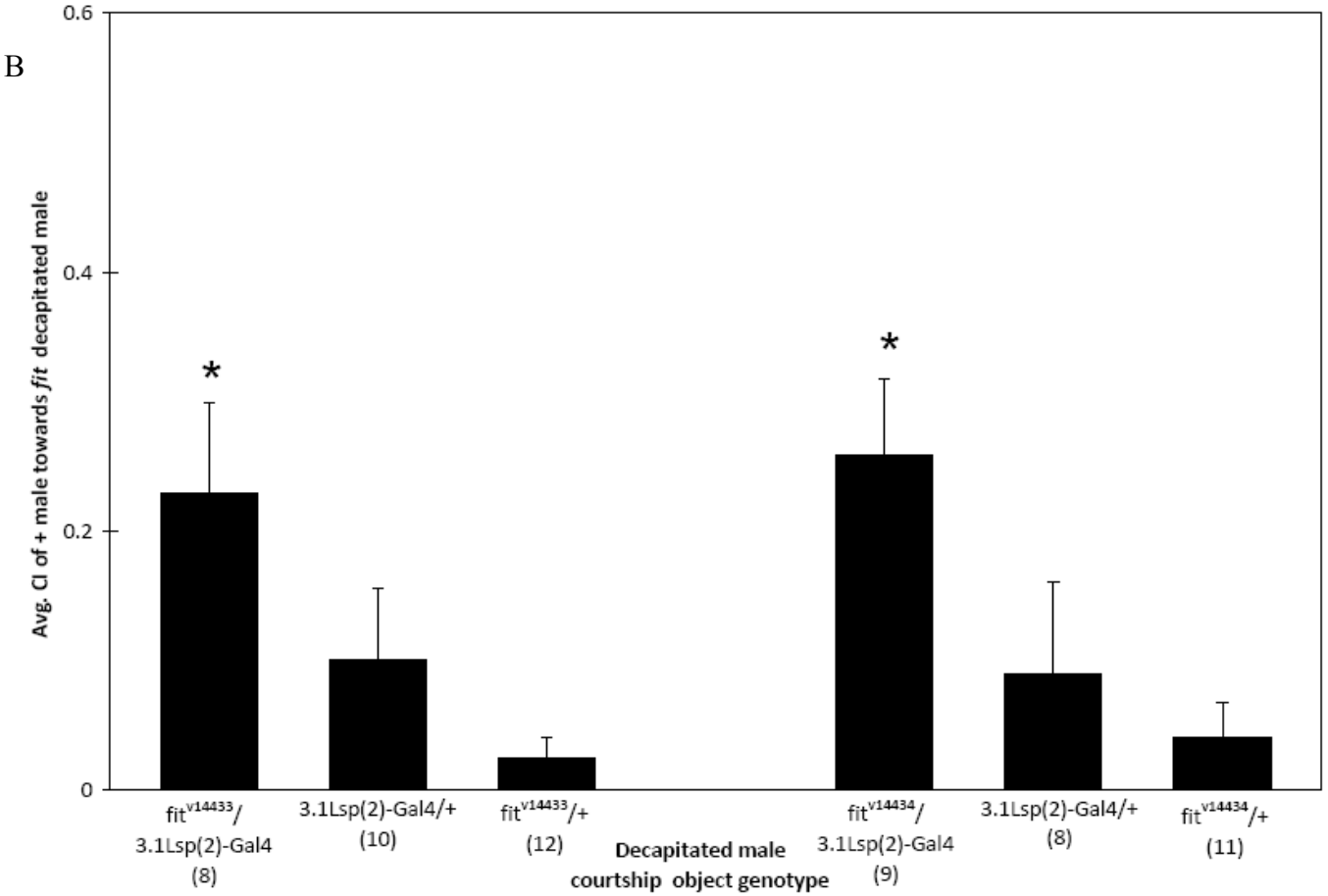


Figure 20. Continued

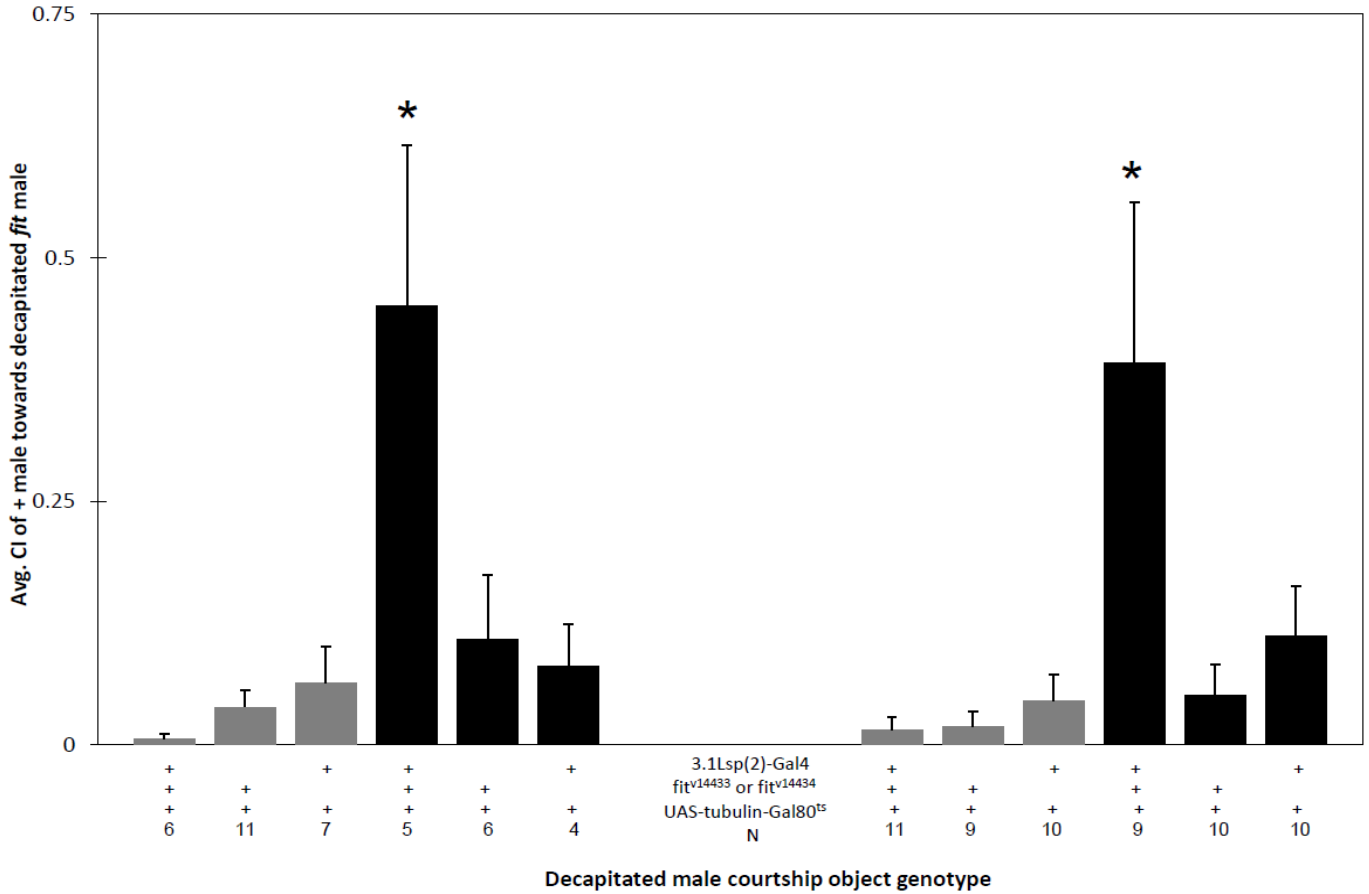


Figure 21. Repression of male-male courtship requires *fit* expression post-developmentally in the fat body. Expressing the *UAS-fit*-RNAi allele, *fit*^{v14433} or *fit*^{v14434}, in the adult fat body using *3.1Lsp(2)-Gal4* and *UAS-tubulinGal80*^{ts}, under the restrictive (29^{o}C, black bars) temperature significantly (*p<0.05) increased the courtship activity of *CS* males toward *fit* mutant males compared to control courtship objects lacking the driver or *UAS-fit*-RNAi component or compared to males at the permissive temperature (20^{o}C, gray bars).

three treatments (Tables 3 and 7). Because of this courtship- and mating-responsiveness and because *fit* is a SDH target (Fujii & Amrein 2002; Goldman and Arbeitman 2007) that is expressed in adipose tissue (Figures 14 and 15), we evaluated *fit's* role in modulating behavior.

Mutations for *fit* change a male's perception of sensory cues and his attractiveness

We generated two independent knock-out alleles for *fit.* Deletion of *fit* did not affect male courtship activity towards *CS* females (Figure 16). A *fit* mutant male paired with a decapitated *fit* mutant male showed increased courtship activity compared to a control male paired with a decapitated control male (Figure 17). However, when given a choice, *fit* mutant males spent more time directing courtship toward *CS* females than *CS* males (Figure 18). Therefore, *fit* males properly perceive female cues. Male-directed courtship is likely due to the *fit* male's inability to process male inhibitory cues.

Male-male courtship can be caused by the improper identification of courtship objects or elicitation by the male courtship object. Males might choose to court another male if (1) he cannot recognize that the courtship object is a male or (2) if the courtship object is attractive. Therefore we tested different combinations of courters and courtees to parse the two phenotypes. Pairing *fit* mutant male courters with control courtees addressed the bisexual phenotype. The elicitation phenotype was assessed by pairing control male courters with *fit* mutant courtees. Our data show that *fit* is required to repress the bisexual and elicitation phenotypes (Figure 17) and that restoring *fit* expression rescues both phenotypes (Figure 19). Examining the literature on Drosophila

courtship defective mutants revealed that the only other genes known to affect both aspects of male-male courtship are *fru* and *dsx* (Appendix A). The small number of known genes modulating the bisexual and elicitation phenotypes may be due to the fact that most male-male courtship behavioral analyses do not separate the two phenotypes; analyses are usually conducted by examining mutant-mutant pairs.

Fat body expression of *fit* modulates male-male courtship

To understand how *fit* modulates both aspects of male-male courtship, we wanted to address which tissues required *fit* expression to repress these behaviors. One tissue of interest is adipose tissue, known as the fat body. *fit* is expressed in the fat body (Figures 14 and 15) and recent experiments have shown that fat body-expressed genes modulate behavior (Dauwalder *et al.* 2002; Fujii and Amrein 2002; Lazareva *et al.* 2007; Fujii *et al.* 2008; Benito *et al.* 2010; reviewed in Dauwalder 2008). Decreasing *fit* expression in the male fat body resulted in increased male-male courtship (Figures 20 and 21) and reducing *fit* in the adult male fat body caused increased courtship elicitation (Figure 21). We are currently examining how reduced post-developmental fat body expression of *fit* affects the bisexual phenotype. Whether or not fat body expression of *fit* is sufficient to repress male-male courtship is also being tested.

Since the fat body is a secretory tissue (reviewed in Schlegel and Stainier 2007), secretion from the fat body likely affects neuronal signaling. Thus fat body-expressed genes may impact courtship behavior, particularly the ability to process sensory cues. Fat body signaling is also tied to oenocyte signaling and its role in pheromone synthesis

(Dellerac *et al.* 2000; Marcillac *et al.* 2005; Ueyama *et al.* 2005; Chertemps *et al.* 2006; Krupp *et al.* 2008; Billeter *et al.* 2009). Therefore, *fit* signaling may function in maintaining the appropriate pheromonal profile.

The role of *fit* in properly recognizing courtship objects or regulating the appropriate hydrocarbon profiles could be attributed to the ability of fat-body expressed factors to interact with multiple tissues. We are also evaluating *fit's* direct involvement in the nervous system in modulating male-male courtship. As we determine if and where Fit is secreted and what proteins Fit interacts with, we can better understand these tissue-specific requirements for modulating behavior.

CHAPTER V

GENOME-WIDE EXPRESSION CHANGES OCCUR IN MATED *Drosophila melanogaster* MALE HEADS

Introduction

Behavior involves the perception and processing of sensory information into a signaling cascade that mediates physiological and motor outputs. This complex process is influenced by an organism's environment, genetic make-up and nervous system function. Social interactions influence an organism's behavior (Siegel and Hall 1979; Gailey *et al.* 1985; Ueda and Kidokoro 2002; Dukas and Mooers 2003; Yurkovic *et al.* 2006), and these behavioral changes are associated with alterations in morphology (Stewart and McLean 2004; Mori *et al.* 2005; Kozorovitskiy *et al.* 2006; Technau 2007) and gene expression (Reiser *et al.* 1999; Levine *et al.* 2002; Mehren and Griffith 2004; Shen *et al.* 2004; Anseloni *et al.* 2005; Burmeister *et al.* 2005; Murata *et al.* 2005; Kozorovitskiy *et al.* 2006; Carney 2007). However, the mechanisms mediating the changes are unclear. As we work to understand the genome-wide transcriptional responses to behavior, we can clarify the regulatory and intracellular processes governing nervous system function and behavior.

Therefore, we are studying reproductive behaviors in the genetically tractable *Drosophila melanogaster,* which exhibit stereotypical mating behaviors (reviewed in Greenspan 1995; Greenspan and Ferveur 2000) regulated by genetics (reviewed in Tompkins 1984; Billeter *et al.* 2002) and social interactions (Dukas and Mooers 2003;

Siwicki and Ladewski 2003; Ellis and Carney 2009; reviewed in Ewing 1983; Greenspan and Ferveur 2000; Mehren *et al.* 2004). The sex-determination gene hierarchy is the major regulator of Drosophila reproduction (reviewed in Cline 2005; Shirangi and McKeown 2007). Components of this pathway affect sexually dimorphic development, including the neural circuitries necessary for sex-specific courtship behaviors (Finley *et al.* 1997; Demir and Dickson 2005; Kimura *et al.* 2005; Manoli *et al.* 2005; Stockinger *et al.* 2005). However, the behavioral functions of only a few of the downstream target genes of the hierarchy are known (Burtis *et al.* 1991; Cann *et al.* 2000; Kopp *et al.* 2000; Dauwalder *et al.* 2002; Fujii and Amrein 2002; Drapeau *et al.* 2003; Arbeitman *et al.* 2004; Goldman and Arbeitman 2007; Lazareva *et al.* 2007Fujii *et al.* 2008; Dalton *et al*. 2009).

By combining behavioral assays with microarray technology, it is possible to assess behaviorally-induced gene expression changes on a genome-wide scale (Ceriani *et al.* 2002; Toma *et al.* 2002; Anholt *et al.* 2003; Dubnau *et al.* 2003; Lawniczak and Begun 2004; Mackay *et al.* 2005; Carney 2007; Ellis and Carney 2009) to find loci regulating or regulated by behavior, including sex-determination hierarchy target genes. Expression profiles of females differ as a consequence of courtship and mating experience, and these changes can be detected for several hours after mating (Lawniczak and Begun 2004; McGraw *et al.* 2004; Mack *et al.* 2006; McGraw *et al.* 2008).

During courtship and mating, the male is inundated with sensory information that must be interpreted so that the appropriate signals are sent throughout the body for a successful mating. Prior work in our lab demonstrated that males rapidly alter gene

expression at the whole-animal level during courtship (Carney 2007; Ellis and Carney 2009). Next, we focused on changes occurring in the male head as a result of mating since these changes likely affect function of the nervous system and other reproductively important tissues to promote reproductive success. Our study demonstrates that courtship culminating in mating affects gene expression patterns in male heads and that many of the gene products are expressed in non-neural tissues that may play important modulatory roles in neural function and behavior.

Materials and Methods

Microarray Analysis

The wild-type *Canton-S (CS)* strain was isogenized to reduce genetic variation and the isoline was kept at 25^{o}C on a 12-hr light/dark cycle. Twenty or fewer virgin *CS* males were aged collectively for 3 days at 25^{o}C. On day 4, individual males were aspirated into vials. Virgin females were collected and aged in groups of 20 or fewer flies for 4 days at 25^{o}C.

On day 5, males were equally divided into two treatment groups. One group, referred to as "mated males", consisted of individual males that were placed with a female for courtship and mating, while the second group of males ("control males") was mock exposed to a female. For the first group, a single, aged virgin female was aspirated into each male's vial. Control males were treated identically except that no female was transmitted during the aspiration process. One male from each group was tested at the same time to allow for statistical comparisons.

Upon completion of mating, females were removed from the vials. Males from both treatment groups were quick frozen 2 hrs later and stored at -80^{o}C for future RNA extraction; only pairs for which the mated male had a mating latency less than 30 min and mating duration of 18-30 min were collected for RNA extraction. Seventy-four percent of mated males tested met this requirement. All procedures were conducted at the same time each day to control for circadian effects.

Head tissue was separated from the remaining body by vortexing quick-frozen flies. Male heads were assigned to one of ten groups (30 heads in each group; 5 mated and 5 control RNA preparations) so that control and mated samples collected together could be analyzed by paired statistical comparisons. Following standard protocols, total RNA from head tissue was extracted in Trizol (Invitrogen, Carlsbad, CA, USA) and RNA preparations from 10 groups (5 control and their corresponding mated groups) were sent to the University of Kentucky MicroArray Core Facility for labeling and hybridization to Affymetrix Drosophila 2.0 Genome Arrays following standard Affymetrix (Santa Clara, CA, USA) protocols.

Expression values were generated similarly to previous experiments (Carney 2007; Ellis and Carney 2009). We used dChip's PM (perfect match between the probe and target sequence) and PM-MM (one nucleotide between the probe and target sequence is mismatched) algorithms (Li and Wong 2001), as well as those implemented by GCOS (MAS 5.0, Affymetrix), R (GCRMA, R Development Core Team 2006), and GeneSpring (Agilent, Santa Clara, CA, USA). For the dChip algorithms, expression values were only considered if greater than 50; for the other 3 methods, expression

values were required to be greater than 100. To test for significance, we used Cyber-T's Bayesian *t*-test analysis (Baldi and Long 2001). Candidate mating-responsive genes included those whose expression differed significantly ($p<0.001$) between control male heads and mated male heads for at least 3 expression value data sets and had a false discovery rate less than 0.05 (Storey and Tibshirani 2003). With such stringent criteria, we did not specify a particular fold change cut-off value.

In order to determine if particular GO terms (molecular functions and biological processes) were overrepresented in our up-regulated and down-regulated data sets, we utilized the Fisher's exact test. The up- and down-regulated lists were compared to all genes represented in the Drosophila 2.0 Genome Array. Significance was determined at the standard p-value<0.05.

Real-time PCR

To confirm the microarray results, Real-time PCR was performed on independent samples that were collected as described above but were not used in the microarray analysis. polyA$^+$ RNA was isolated using the Oligotex mRNA mini kit (Qiagen, Netherlands) from 5 mated male RNA samples and their corresponding control male RNA samples (30 heads per RNA preparation). cDNA was synthesized using the SuperScript First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). We designed primers to amplify 10 up-regulated and 3 down-regulated genes, choosing genes that are predicted to be enriched in brain, fat body or both tissues based upon FlyAtlas expression data (Chintapalli *et al.* 2007). When possible, primer pairs were

designed across introns to control for amplification specificity. Genes that are expressed at low levels in the head (Chintapalli *et al.* 2007) were not tested.

Using the SYBR Green PCR Mastermix (Applied Biosystems, Foster City, CA, USA), 2μL of a 1:4 dilution of each template was run in triplicate in the ABI7500 (Applied Biosystems, Foster City, CA, USA) using default parameters. Control reactions lacking template and controls with template but without Reverse Transcriptase were used. Primer-specific amplification was determined by analyzing dissociation curves for each primer pair.

mRNA levels were determined by the Relative Standard Curve Method (Applied Biosystems, Foster City, CA, USA), and candidate gene transcript levels were normalized to *rp49* transcript levels. Normalizing the mated male transcript levels to the control male transcript levels generated a relative fold change. We also analyzed trends in the average relative transcript levels of each treatment (control & mated) using the two-tailed *t*-test. Secondary RT-PCR analysis confirmed increased expression of *CG6188* and decreased expression of *alpha Esterase-2*.

In situ hybridization

Digoxigenin (DIG)-labeled RNA probes were made from cDNA clones for five candidate genes with predicted fat body expression following the manufacturer's standard protocol (Roche, Nutley, NJ, USA). The genes and their corresponding cDNA clones were *CG4825* (LD10327), *CG8449* (GH10459), *CG13360* (LP09811), *bubblegum* (*bgm*) (GM14009) and *Prx2540-2* (RH69586). Expression of *Prx2540-2* is

regulated by *fruitless* (*fru*) (Goldman and Arbeitman 2007), a regulatory component of the sex-determination hierarchy.

Antisense and sense probes were hydrolyzed into 200bp fragments and *in situ* hybridization to male brains, head carcass and abdominal cuticle was performed as described in Arbeitman *et al.* (2004).

Courtship assays

All flies were kept on a 12-hr light/dark cycle at 25°C. P-element insertion mutations in *Jhe* and *cricklet* (*clt*) were obtained from the Bloomington Drosophila Stock Center ($clt^{BG01317}$) and the Exelixis Collection at Harvard Medical School (Jhe^{e01859}). Each P-element was crossed into the *CS* background to generate a genetically similar control that had one wild-type copy of *Jhe* or *clt*. To test for a genetic interaction between *Jhe* and *clt*, the two insertion strains, Jhe^{e01859} and $clt^{BG01317}$, were crossed to generate transheterozygous flies containing a single P-element insertion in each gene (Jhe^{e01859} +/+ $clt^{BG01317}$). Virgin P-insertion or control males were collected and stored individually for 4 to 5 days; virgin *CS* females were aged collectively for 3 to 5 days.

Behavioral assays were conducted at 22°C under dim red light conditions (forcing the males to rely on other sensory modalities besides vision) and recorded with a digital camcorder so that subsequent analyses could be performed. To analyze courtship behavior, a male was aspirated into a mating chamber (diameter=1 cm) and a virgin *CS* female was introduced 2 min later. The pair was video recorded for 10 min. The courtship index (CI; percent of time the male spent performing courtship during the

initial 10 min of observation) and courtship latency (time until courtship occurs) were calculated. CI values were arcsine transformed for statistical analysis. Two-tailed *t*-test comparisons between homozygous mutants and controls were calculated to determine significance ($p<0.05$). Jhe^{e01859} +/+ $clt^{BG01317}$ males were compared to both controls.

Fertility assays

The ability of a male to mate with multiple *CS* females and the fecundity of these matings was also assessed. *Jhe* and *clt* mutants and heterozygous controls, as well as *CS* virgin females, were collected and aged as described for the courtship assay. Under red light, a male was aspirated into a mating chamber, followed by a *CS* virgin female. The male was given 2 hrs to mate with the female. If mating occurred, the female was placed in a vial with food to measure fecundity (number of eggs laid and number of adult offspring) and the male was placed in a new mating chamber. A second *CS* virgin female was aspirated into the new chamber and the pair was given 2 hrs to mate. If the second mating occurred, the female was placed in a vial for later progeny counts, and the male was moved to another chamber for mating with a third and final female. The third mated female was also kept for further analysis.

For the first mating bout, all 10 $clt^{BG01317}$ males mated, while only 3 of the 10 males mated with the second female and none of the 3 males mated with the third female. Eight out of 13 $clt^{BG01317/+}$ males mated with the first female, 5 of those 8 males mated with the second female and 4 of the remaining 5 males mated with the third female. Jhe^{e01859} males only mated with the first female (6 out of 9 males). However, 9

of 10 Jhe^{e01859}/+ males mated with the first female, 7 of those 9 males mated with the second female and 4 of the 7 males mated with the third female. For the transheterozygous $clt^{BG01317}/Jhe^{e01859}$ males, 7 of 12 mated with the first female, 4 of 7 males mated with the second female and 2 of the 4 males mated with the third female.

The mating latencies and durations for each of the 3 possible matings were measured and significance was determined by Univariate ANOVA analysis using genotype and mating trial as fixed variables with Tukey's post-hoc analysis (SPSS). Males that did not mate within the 2 hr window were scored as being unsuccessful. Using linear regression, we assessed the significance ($p<0.05$) of genotype and mating bout on mating success.

For 6 days following the assay, each mated female was transferred to a new vial and the number of eggs laid in each vial was determined. Vials were maintained at 25^{o}C for 18 days to allow for a count of the total number of adult progeny. Significant effects of genotype and trial on mating latency or duration were measured by the Univariate ANOVA and Tukey's analysis. We also measured the significance of genotype, mating bout and day of egg laying on the male's fecundity (Univariate ANOVA and Tukey's post-hoc analysis). Fecundity was measured by the total number of eggs laid and by the arcsine transformed ratio of adult offspring to eggs laid.

Results

Mating causes expression changes in male heads

Gene expression levels change rapidly as males court females (Carney 2007). To

determine the effects of courtship culminating in mating on male gene expression, we compared transcriptional profiles of males that mated with a female (mated males) to those that were not presented with a female (control males). Labeled samples from control and treatment groups were hybridized to Drosophila Genome 2.0 Arrays (Affymetrix, Santa Clara, CA, USA), which are based on the Flybase 3.1 annotation, targeting nearly 18,500 transcripts.

In the current study we focused on head expression, rather than whole body expression (Carney 2007; Ellis and Carney 2009), to identify gene expression changes in the nervous system and other tissues within the head (such as sensory systems and fat body) that likely modulate reproduction. We isolated male heads (rather than dissecting out the brains) since accumulating evidence from our lab (Carney 2007; Ellis and Carney 2009) as well as from other published studies (Dauwalder *et al.* 2002; Fujii and Amrein 2002; Lazareva *et al.* 2007; Fujii *et al.* 2008; reviewed in Dauwalder 2008) indicate that head tissues, such as the fat body, likely also have important modulatory functions in behavior. To have the potential to identify gene expression changes in these tissues as well, we elected to assay the entire male head for alterations in gene expression patterns in response to mating.

We used five algorithms to extract expression values from each array and performed paired *t*-test comparisons between mated male heads and control male heads. Using this strategy we identified 47 mating-responsive genes (See Materials and Methods). Two hrs after mating with a female, males significantly up regulated 25 genes (Table 7) and down regulated 22 genes (Table 8). Such changes are not likely to be

activity-dependent since control males had locomotor levels similar to males that courted females (*t*-test; $p>0.05$). Genes representing a variety of molecular functions and biological processes, determined by Gene Ontology (GO) annotations, were present in our data set. Several molecular functions and biological processes are over-represented in the up-regulated or down-regulated data sets compared to the Drosophila genome (Tables 7 and 8; Fisher's exact test, $p<0.05$).

Table 7. Candidate genes up regulated 2 hrs after mating

Gene identifier	Gene name	Avg. fold change	GO Molecular function	GO Biological process
CG2163	*polyA-binding protein II* (*Pabp2*)	1.4	Poly(A) binding*	mRNA polyadenylation*
CG4288		1.28	High affinity inorganic phosphate: sodium symporter activity*	Transport
CG4501	*bubblegum* (*bgm*)	1.38	Long-chain fatty acid-CoA ligase activity	Long-chain fatty acid metabolic process*
CG4825	*Phosphatidyl-serine synthase*	1.22	CDP-diacylglycerol-serine O-phosphatidyl-transferase activity*	Phosphatidylserine biosynthetic process*
CG5527		1.23	Endothelin-converting enzyme activity*	Proteolysis
CG5618		1.14	Dipeptidyl-peptidase III activity*	Proteolysis
CG6188		1.64	Glycine N-methyltransferase activity*	Unknown
CG6342	*Iron regulatory protein 1B* (*Irp-1B*)	1.26	Iron ion binding	Regulation of translational initiation by iron*
CG8425	*Juvenile hormone esterase* (*Jhe*)	1.86	Juvenile-hormone esterase activity*	Juvenile hormone catabolic process*

Table 7. Continued

Gene identifier	Gene name	Avg. fold change	GO Molecular function	GO Biological process
CG8449		1.28	Rab GTPase activator activity	Regulation of Rab GTPase activity*
CG9989		1.52	Endonuclease activity*	Unknown
CG11765	*Peroxiredoxin 2540* (*Prx2540-2*)	1.2	Antioxidant activity*	Unknown
CG12116		1.22	Sepiapterin reductase activity*	Metabolic process
CG13360		1.28	Unknown	Unknown
CG13607		1.23	Unknown	Unknown
CG13965		1.35	Unknown	Unknown
CG16772		1.5	Unknown	Unknown
CG16901	*squid* (*sqd*)	1.25	mRNA binding	Oocyte axis determination*
CG17364		1.67	GTP binding	Microtubule-based process
CG17820	*female-specific independent of transformer* (*fit*)	1.4	Unknown	Unknown
CG18262		1.3	Zinc ion binding	Unknown
CG30026		1.42	Unknown	Unknown
CG30095		1.86	Oxidoreductase activity	Metabolic process
CG30084	*Z band alternatively spliced PDZ-motif protein 52* (*Zasp52*)	1.38	Protein binding	Unknown
CG33486	*asparagine synthetase*	1.28	Asparagine synthetase (glutamine-hydrolyzing) activity*	Asparagine biosynthetic process*

Twenty-five genes are significantly ($p<0.001$) up regulated in male heads 2 hrs after mating when compared to control male heads. Over-represented (*$p<0.05$) molecular functions and biological processes were determined by Fisher's exact test.

Table 8. Candidate genes down regulated 2 hrs after mating

Gene identifier	Gene name	Avg. fold change	GO Molecular function	GO Biological process
CG1897	*Drop* (*Dr*)	-1.5	DNA binding	Central nervous system development
CG2505	*α-Esterase-2* (*α-Est2*)	-1.3	Carboxylesterase activity	Unknown
CG3200	*Rhythmically expressed gene 2* (*Reg-2*)	-1.27	Phosphoglycolate phosphatase activity*	Metabolic process
CG3926	*Serine pyruvate aminotrans-ferase* (*Spat*)	-1.34	Serine-pyruvate transamine activity	Glyoxylate catabolic process*
CG4105	*Cytochrome P450-4e3* (*Cyp4e3*)	-1.3	Electron carrier activity	Unknown
CG5840		-1.34	Pyrroline-5-carboxylate reductase activity*	Proline biosynthetic process*
CG6806	*Larval serum protein 2* (*Lsp2*)	-1.28	Nutrient reservoir activity*	Transport
CG7224		-1.16	Unknown	Unknown
CG7390	*senescence marker protein-30* (*smp-30*)	-1.36	Unknown	Unknown
CG8112		-1.42	Sterol O-acyltransferase activity*	Unknown
CG8846	*Thor*	-1.26	Eukaryotic initation factor 4E binding	Immune response
CG9416		-1.25	Sequence-specific DNA binding	Regulation of transcription
CG9733		-1.6	Trypsin activity*	Proteolysis
CG11909		-1.42	α-glucosidase activity	Carbohydrate metabolic process
CG11919		-1.36	ATP binding	Peroxisome organization and biogenesis*
CG16898		-1.68	Unknown	Unknown

Table 8. Continued

Gene identifier	Gene name	Avg. fold change	GO Molecular function	GO Biological process
CG18003		-1.36	Glycolate oxidase activity*	Metabolic process
CG30489	*Cyp12d1-p*	-1.3	Electron carrier activity	Unknown
CG31075		-1.26	Aldehyde dehydrogenase (NAD) activity*	Pyruvate metabolic process*
CG31628	*adenosine 3* (*ade3*)	-1.28	Phosphoribosylamine-glycine ligase activity*	Purine base biosynthetic process*
CG31689		-1.25	ATPase activity	Unknown
CG33462		-4.08	Trypsin activity*	Proteolysis

Average fold changes, molecular functions and biological processes are shown for 22 genes that are significantly ($p<0.001$) down regulated in male heads 2 hrs after mating. *$p<0.05$, Fisher's exact test.

Verification of microarray results by independent Real-time PCR (RT-PCR)

To confirm the microarray results, we performed RT-PCR analysis on independently collected mated and control male head RNA samples. We tested a subset of genes whose expression levels changed significantly in mated male heads compared to control male heads. Eight out of 10 up-regulated genes and 2 out of 3 down-regulated genes had the expected directional change (Table 9).

Expression of candidate genes is not restricted to the brain

We hypothesized that examining gene expression in head tissue instead of whole bodies would uncover genes that function in reproduction by regulating nervous system signaling. This could be via direct effects on neural gene expression or by effects on other tissues in the head that receive or respond to courtship and mating signals. We

found that expression of many mating-responsive genes is enriched in the head but not the brain (Table 10) (Chintapalli *et al.* 2007), indicating expression occurs outside of the brain. While some of the genes are expressed in the eye, others appear enriched in tissues other than the brain and eye.

Table 9. Confirmation of microarray results by RT-PCR

Gene identifier	Gene symbol	Relative fold change±SEM	Avg. relative expression level in control male heads±SEM	Avg. relative expression level in mated male heads±SEM
CG5618		2.02±0.49*	0.36±0.09	0.74±0.18
CG6188		1.94±0.26*	2.25±0.42	4.38±0.58
CG8449		1.35±0.15*	1.24±0.26	1.68±0.18
CG16772		4.07±1.55	6.86±1.72	27.94±10.61
CG30026		2.23±0.35*	4.36±0.84	9.74±1.53
CG4501	*bgm*	4.47±1.11*	1.42±0.31	6.37±1.57
CG6342	*lrp-1B*	1.42±0.12	1.09±0.18	1.55±0.13
CG11765	*Prx2540-2*	1.23±0.12*	0.47±0.08	0.58±0.06
CG2505	*αEst2*	-1.26±0.15	2.84±0.59	2.25±0.42
CG7390	*smp-30*	-1.69±0.16*	1.28±0.45	0.77±0.2

*Indicates a significant ($p<0.05$) difference between the average relative expression level in control male heads and mated male heads. SEM=Standard error of the mean.

One possibility is that they are expressed in an adipose tissue called the fat body that surrounds the brain and is implicated in modulating courtship behavior (Dauwalder *et al.* 2002; Fujii and Amrein 2002; Lazareva *et al.* 2007; Fujii *et al.* 2008; reviewed in Dauwalder 2008). Data showing that mating-responsive genes enriched in the head are also enriched in the adult fat body (Table 10) (Chintapalli *et al.* 2007) support this hypothesis. *In situ* hybridization confirmed that several mating-responsive loci are expressed in male fat body tissue (Figure 22).

FlyAtlas data indicate that the fat-expressed genes *CG13360, bubblegum* (*bgm*) and *Prx2540-2* are expressed at very low levels in brains, while *CG8449* and *CG4825* are expressed at low to moderate levels in the brain (Chintapalli *et al.* 2007). By *in situ* we did not detect brain expression of the five assayed transcripts (Figure 22 & data not shown), although we cannot rule out the possibility that low levels of message are present.

Table 10. Candidate genes are enriched in head tissue other than the brain, including adult adipose tissue

	Total no. of genes	Head	Brain	Eye	Fat body
Up regulated	25	18	4	9	16
Down regulated	22	20	2	12	18

Data was compiled from FlyAtlas (Chintapalli *et al.* 2007).

Juvenile hormone esterases are important for male reproductive behaviors

We hypothesized that if a gene is up regulated after mating, that gene likely affects some aspect of reproductive behavior. Therefore, we assayed the percent of time a male spent courting a female in a given interval, known as the courtship index (CI), of candidate gene mutants. A *Jhe* P-element insertion, Jhe^{e01859}, resulted in significantly reduced CI values (Figure 23). Though *Jhe* males court females less vigorously, they perform standard courtship steps, culminating in copulation.

In addition to *Jhe* there are three other candidate juvenile hormone esterase genes in the Drosophila genome (Campbell *et al.* 2001). One of the genes, *cricklet* (*clt*), also had an available P-element insertion, so we tested $clt^{BG01317}$ mutants to see if they had a similar phenotype to *Jhe* mutants. We found that *clt* mutants also have decreased CIs

relative to controls (Figure 23). There is also a strong genetic interaction between *Jhe* and *clt*. Transheterozygous mutant males had significantly reduced courtship compared to single insertion controls (Figure 23).

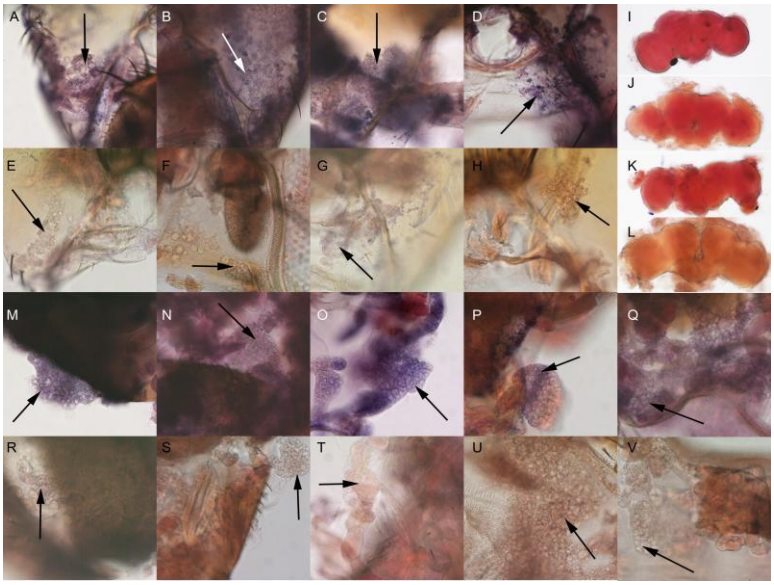


Figure 22: Candidate genes are expressed in fat tissue. Antisense (A-D,I,K,M-Q) or sense (E-H,J,L,R-V) RNA probes were designed to cDNA clones for *CG4825* (A,E,M,R), *CG8449* (B,F,I,J,N,S), *bgm* (C,G,O,T), *Prx2540-2* (D,H,K,L,P,U), and *CG13360* (Q,V),. *In situ* hybridization to whole-mount tissue shows candidate gene expression in male *CS* fat body tissue (arrows) on head (A-H) and abdominal (M-V) cuticle.

We further examined the mating kinetics of *Jhe* and *clt* mutants, expecting that the genes might function in regulating mating latency and duration as well as priming the male for subsequent mating encounters. Though *Jhe* and *clt* males mate with females, they had a significant ($p<0.05$) increase in mating latency (Figure 24) (ANOVA, genotype $p<0.05$, trial $p>0.05$), while mating duration was unaffected. The increased mating latency was not dependent on the mating trial (1^{st}, 2^{nd} or 3^{rd}). However, as we increased the number of mating attempts, the mating success (as measured by the

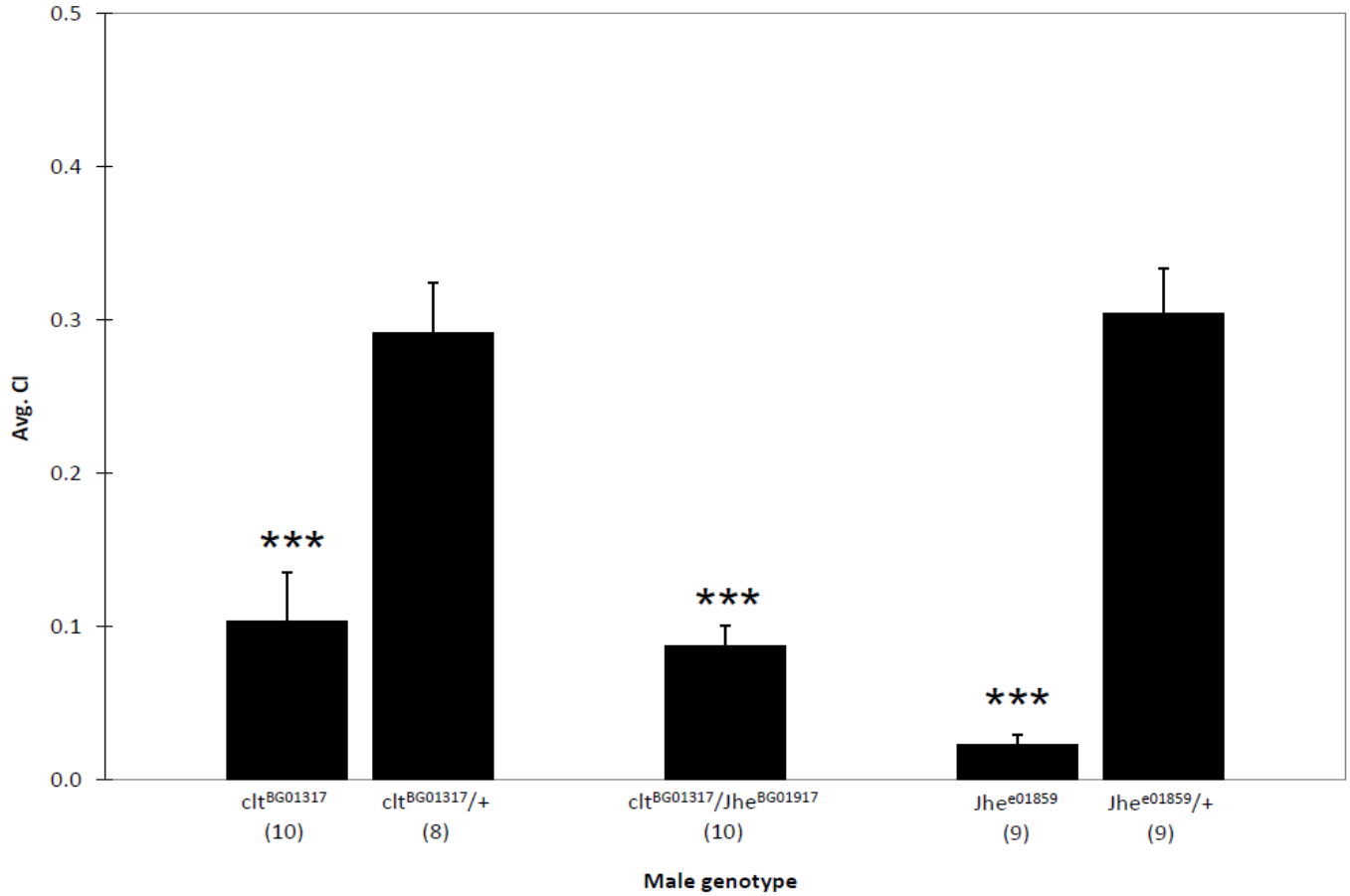


Figure 23. *Jhe* and *clt* mutants reduce courtship toward females. Mutant males homozygous for P-element insertions in *Jhe* or *clt* show reduced courtship (***p<0.001) under red light compared to sibling heterozygous controls under the same condition. Jhe^{e01859} +/+ $clt^{BG01317}$ mutant males showed significant reductions in courtship compared to either heterozygous control.(N)=sample size. Error bars are SEM.

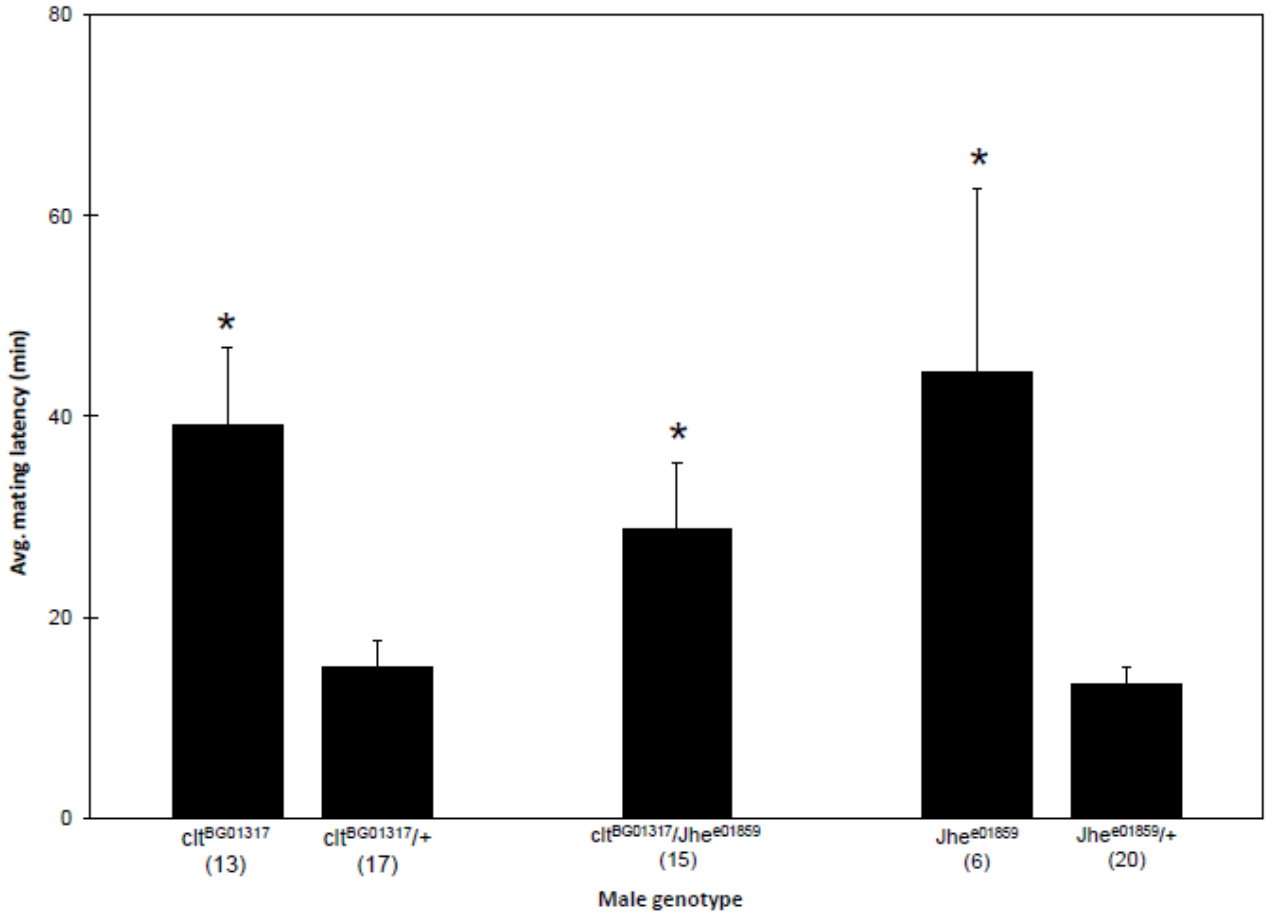


Figure 24. Mating latency is increased in *Jhe* and *clt* mutants. Homozygous and transheterozygous mutant males had significantly (ANOVA $p<0.01$, Tukey's $*p<0.05$) increased mating latencies toward *CS* virgin females regardless of the mating bout (1st, 2nd, or 3rd); therefore, overall average mating latencies are shown.

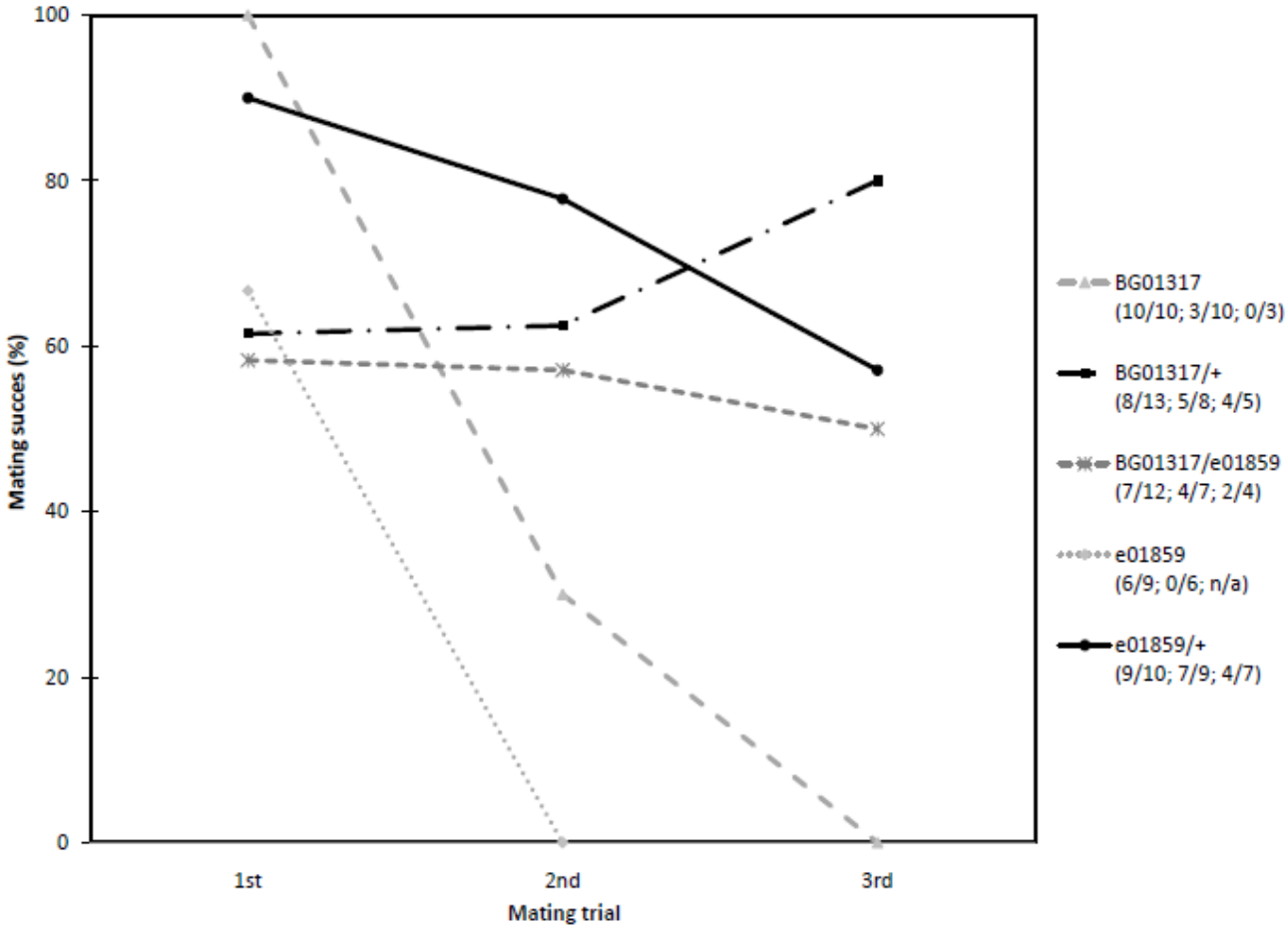


Figure 25. Mating success decreases in *Jhe* and *clt* mutants. *Jhe*/+ and *clt*/+ control males mated with 3 females in succession, while experimental *Jhe* and *clt* mutant males significantly (Binary Logistic Regression, genotype $p<0.01$, trial $p<0.0001$, interaction $p<0.0001$) decreased their mating success with the 2nd and 3rd females.

act of copulation) of *Jhe* and *clt* mutant males was significantly reduced (Figure 25) (Binary Logistic Regression, genotype $p< 0.01$, trial $p<0.0001$, interaction $p<0.0001$). Females mated to *Jhe* or *clt* mutant males lay equivalent numbers of eggs regardless of the mating trial and day of egg laying (ANOVA, genotype $p>0.05$, trial $p>0.05$, day $p>0.05$), and neither *Jhe* nor *clt* mutant females had detectable fertility defects.

Discussion

Genome-wide response to mating

The complex reproductive behaviors exhibited by Drosophila require the interaction between genetics and environment. Courtship is an innate and stereotypical process under control of the somatic sex-determination hierarchy and is influenced by social interactions. Courtship and mating elicit gene expression changes in females (Lawniczak and Begun 2004; McGraw *et al.* 2004; Mack *et al.* 2006; McGraw *et al.* 2008), and courtship affects transcript profiles in males (Carney 2007; Ellis and Carney 2009). The female post-mating effects occur rapidly (within minutes) or can be detected several hours after mating (Lawniczak and Begun 2004; McGraw *et al.* 2004; Mack *et al.* 2006; McGraw *et al.* 2008). Within 5 min of courtship, whole-animal gene expression profiles also change rapidly in males (Carney 2007; Ellis and Carney 2009). In this study we expand on our earlier studies in males to show that courtship culminating in mating causes changes in gene expression in the male head as well. Expression levels likely change rapidly in response to sensory cues received during courtship, while the physiological changes from mating (DiBenedetto *et al.*1990) may

mediate long-term expression level changes in the nervous system or elsewhere in the fly that can feed back to the nervous system.

The expression profile of a 5 min courting male differs from that of a 2 hr post-mating male. This is not surprising since we expected that the process of mating would have major effects on male physiology that would be reflected in altered transcriptional profiles. Of the 47 genes with altered expression 2 hrs after mating, only 1 gene, *fit,* is also up regulated in males after 5 min of courtship (Carney 2007). *CG16772* is up regulated 2 hrs after mating but is down regulated during 5 min of courtship (Carney 2007). *CG16772* is one of several fat body-expressed immune response genes down regulated during courtship, possibly to allow energetic resources to be directed toward offspring production rather than immunity (Carney 2007; Ellis and Carney 2009). After mating, expression of *CG16772* may increase because contact with a female increases the likelihood of encountering a pathogen.

The fact that few genes overlap between these data sets is not surprising since we assayed different time points (5 min or 2 hrs), different tissues (whole bodies in previous studies versus heads in this study) and different behaviors (courtship alone versus courtship culminating in mating). We also used different approaches for analyzing the data due to the differences in experimental design for each test. The analysis strategies provide us a conservative estimate of the transcripts affected by courtship and mating.

Gene expression in adipose tissue

The fat body is secretory tissue (reviewed in Schlegel and Stainier 2007) whose

effects on fly reproductive behavior have previously been described (Dauwalder *et al.* 2002; Fujii and Amrein 2002; Lazareva *et al.* 2007; Fujii *et al.* 2008; reviewed in Dauwalder 2008). The majority of mating-responsive genes are expressed in adult adipose tissue (fat body) (Table 10), and we analyzed a subset of the genes to show that they are expressed in adipose tissue surrounding the brain (Figure 22). Of the 25 genes up regulated by courtship and mating, 14 are detectable (signal strength greater than 20) in brain and 21 genes are detectable in fat body based upon a microarray analysis of adult mRNA expression levels (Chintapalli *et al.* 2007). Of these 25 up regulated genes, 16 are enriched in the fat body relative to other adult tissues (Table 10).

Other studies also indicated that several mating-responsive genes identified in our study are expressed in the fat body surrounding the brain. *Larval serum protein 2* (*Lsp2*) is expressed in the head fat of both sexes (Benes *et al.* 1990). *fit* is expressed in the head fat of females and originally was named based upon its high expression in females under the control of *Sex-lethal* (Fujii and Amrein 2002), which is the initial regulatory gene in the somatic sex-determination hierarchy. *fit* also is expressed in virgin male fat body at low levels (Figure 14), and *fit* expression increases in response to courtship as well as mating (Carney 2007; Ellis and Carney 2009) (Figure 14).

A third mating-responsive gene, *Juvenile hormone esterase* (*Jhe*), is also expressed in adipose tissue (Klages & Emmerich 1979; Renucci 1986; Wroblewski *et al.* 1990; Shanmugavelu *et al.* 2000; Hinton & Hammock 2003; Bai *et al.* 2007; Kamimura *et al.* 2007; Munyiri and Ishikawa 2007; Anand *et al.* 2008; Liu *et al.* 2008). We have shown that *Jhe* is required for male reproductive behaviors. *Jhe* and three closely related

esterase genes (*clt*, *Jhedup*, and *CG7529*) have juvenile hormone esterase activity *in vitro*, although *Jhe* was predicted to be the physiologically active enzyme (Crone *et al.* 2007). Juvenile hormone esterases (JHEs) together with juvenile hormone epoxide hydrolases (JHEHs) hydrolyze Juvenile hormone (JH) to regulate JH levels (Campbell *et al.* 1992; Campbell *et al.* 1998). Since homozygous *Jhe* and *clt* mutants as well as transheterozygous mutants have similar negative effects on male reproductive behavior (Figures 23-25), it appears likely that both JHE and CLT have juvenile hormone regulatory function *in vivo*.

Much of our understanding of physiological functions of JH comes from studies investigating its function during development (reviewed by Flatt *et al.* 2005). However, JH also has important post-developmental functions such as promoting accessory gland protein (Acp) synthesis (Wolfner *et al.* 1997b). During mating Acps are transferred along with sperm, to the female (Wolfner, 1997), and the transfer of Acps triggers male synthesis of new Acps (DiBenedetto *et al.* 1990). Males also transfer Sex-peptide to the female during mating (Kubli 1992; Chen 1996; Wolfner *et al.* 1997a). Sex-peptide increases JH levels in females (Moshitzky *et al.* 1996), which stimulates egg development (Soller *et al.* 1999). However, possible mating-induced changes in male JH levels have not been evaluated.

Jhe expression is responsive to fluctuating JH levels (Kethidi *et al.* 2005), so the mating induced increase in *Jhe* expression identified in our study may be JH dependent. Since ejaculate components must be replenished after mating, we hypothesize that JH levels increase after mating to stimulate Acp synthesis. The increase in JH would

increase *Jhe* expression which would, in turn, negatively regulate JH and JH-induced Acp production.

In addition to its physiological role in regulating Acp synthesis, JH affects reproductive behaviors. JH is necessary for the post-mating response in females (Soller *et al.* 1999), and loss of JH results in decreased courtship activity in males (Wilson *et al.* 2003). Overexpression of the JHE binding protein DmP29, which is expected to decrease JH titers, causes a variety of phenotypes, including increased male-to-male courtship and decreased female receptivity (Liu *et al.* 2008). Decreased expression of DmP29 (which should increase JH titers) causes increased female fecundity but has no obvious effect on male fecundity; male behavior was not examined in animals with decreased DmP29 (Liu *et al.* 2008).

Though the loss of JH disrupts courtship behavior (Wilson *et al.* 2003), our data suggest that an increase in JH, caused by reduction of *Jhe* or *clt*, also disrupts courtship (Figures 23-25). *Jhe* and *clt* deficient males, which likely have increased levels of JH, court less vigorously (Figure 23), have increased mating latencies (Figure 24), and have reduced mating success (Figure 25). However, *CS* females mated to *Jhe* or *clt* males laid similar numbers of eggs compared to *CS* females mated to control males. This situation exemplifies the complex regulation governing behavior and implies that JH levels must be tightly regulated in order to ensure appropriate behavioral and physiological responses.

Together, these results imply that the brain is not the only tissue responding to or regulating post-mating behavior, but that adipose tissue plays a role in this process as

well. In response to mating, a signaling cascade initiated by neurosecretory cells may transmit the signal to the surrounding fat body. The fat body then could perpetuate the signal by secreting factors that influence neuronal or non-neuronal tissues. We hypothesize that expression level changes in the brain alter neuronal signaling either directly or indirectly, which impacts the processing of sensory cues and targets other reproductively important tissues.

We predict that some mating-responsive genes facilitate an increased male mating efficiency for future encounters. Little is known about how repeated matings affect male mating latency, duration or fecundity. Data from our lab indicate that male mating latency decreases due to experience (C. C. Schwedes & G. E. Carney, unpublished results). After his first mating, the male may perceive and process female stimuli more rapidly, may be more appealing to the female, or may be physiologically primed for subsequent matings by replenishment of Acps, sperm or other seminal proteins, resulting in decreased courtship or mating latencies. Alterations in gene expression, such as those described here and in our earlier work (Carney 2007; Ellis and Carney 2009), may contribute to these expected behavioral and physiological changes.

Gene expression in the brain

Although we are particularly interested in the large number of fat-enriched or fat-expressed genes that were identified in this and earlier screens (Carney 2007; Ellis and Carney 2009), we also note that many of the identified transcripts are expressed in brains. Thirteen of the 21 fat-expressed genes up regulated in mated males are also

expressed in brains at detectable levels (Chintapalli *et al.* 2007); a single transcript, *CG4288* is detected in brains but not fat (Chintapalli *et al.* 2007).

Mutants for *bgm*, an enzyme involved in fatty acid metabolism that is expressed in both the brain and fat, have a neurodegeneration phenotype in response to accumulation of long chain fatty acids (Min and Benzer 1999). Another gene that potentially functions in a neurodegeneration pathway is *CG4825*, which responds to changes in polygluatmate (polyQ) levels (Nelson *et al.* 2005). polyQ diseases, including Huntington's Disease, are adult onset progressive neural degeneration diseases caused by the accumulation of glutamate repeats (Zoghbi and Orr 2000).

Cellular homeostasis is important in the maintenance and function of the Drosophila brain. One gene that helps maintain this homeostasis is *Iron regulatory protein 1B* (*Irp-1B*) which encodes a protein that binds to iron-responsive elements (IREs) to regulate iron metabolism (Muckenthaler *et al.* 1998). In addition to affecting cell survival and homeostasis, neural morphology might also be regulated by mating-responsive candidates. Mutants of *Pabp2* show pathfinding and targeting defects in the larval neuromuscular junction (Liebl *et al.* 2006).

Proper function of the nervous system relies on the appropriate cellular architecture, connections and signaling. Behavior requires the sensory systems to perceive the information accurately and transmit such information to the brain for processing. The brain can then transmit the signal to the appropriate output pathways which can modify signaling in tissues such as the fat body or the brain itself. Therefore the establishment and maintenance of the brain (and sensory systems) is vital to the

organism's ability to respond to its environment and experience. The mating-responsive gene *Drop* is involved in nervous system development (Skeath and Thor 2003; Urbach and Technau 2004), and it is possible that *Drop* and other mating-responsive genes act in the development or maintenance of a mated male brain as opposed to a naïve male brain.

Mating-responsive genes and the sex-determination hierarchy

This genome-wide analysis identified known sex-determination hierarchy target genes such as *fit*. Three other mating-responsive genes (*CG16772*, *Prx2540-2* and *CG16898*) (Tables 3 and 4) are also regulated by the sex-determination hierarchy (Goldman and Arbeitman 2007). Transcriptional profiling of mutants for a variety of sex-determination hierarchy genes indicates that *Prx2540-2* and *CG16898* are regulated by *fruitless* (*fru*), while *fit* is downstream of *transformer* (*tra*). *CG16772* may also function downstream of *tra* (Goldman and Arbeitman 2007).

The splicing factor *squid* (*sqd*) is up regulated in mated male heads (Table 7). Interestingly, primary transcripts of the *sqd* locus are sex-specifically spliced in the head as well as the germline, although it is not known if *sqd* splicing is regulated by the sex-determination hierarchy (Telonis-Scott *et al.* 2009). It is possible that *sqd* and other mating-responsive loci function as downstream targets of the sex-determination hierarchy to regulate morphological and behavioral differences between male and female Drosophila. Alternatively, there may be other pathways (such as those that regulate alternative splicing) that function together with the sex-determination hierarchy

to regulate reproductive behavior. We predict that mating-responsive genes also function in other aspects of reproduction; therefore, we propose this transcriptional profiling approach is a powerful strategy for determining the genetic pathways and intracellular processes regulating reproduction, both at the behavioral and physiological levels.

CHAPTER VI

SUMMARY AND CONCLUSIONS

Courtship or mating alter male gene expression profiles

We present the first pieces of evidence that the performance of reproductive behaviors alters gene expression profiles of male Drosophila. Not only do we show that freely behaving male Drosophila have altered gene expression, but we also show that variation in their environment (such as courtship cues from a conspecific versus heterospecific female) rapidly alters their behavior (Figure 4).

These gene expression changes are tissue specific (head versus whole body) and occur rapidly (within 5 or 20 min). Head tissue includes the brain and peripheral nervous system structures (eyes and antennae) and since courtship involves nervous system function within these modalities, we hypothesized that focusing on head tissue would reveal behaviorally important loci.

Insights into fat body modulation of courtship behavior through the first behavioral analysis of *fit*

Analysis of our courtship-responsive genes revealed that many loci are enriched in the head compared to the brain, implying that these candidate genes are enriched in tissues such as the eye, antennae or the fat body (Tables 6 and 10). Fat body expression of our candidate genes (Figures 7, 14, and 22) was particularly intriguing because there is a small body of work demonstrating that the fat body serves functions beyond

metabolism; the fat body also modulates reproductive behaiors (Dauwalder *et al.* 2002; Fujii and Amrein 2002; Lazareva *et al.* 2007; Fujii *et al.* 2008; reviewed in Dauwalder 2008). For example, sexual identity of the fat body affects male courtship behavior; feminization of the adult male fat body results in decreased circulating levels of the male-specific Takeout and reduced male-female courtship activity (Lazareva *et al.* 2007).

Since we have shown that courtship- or mating-responsive loci are expressed in the fat body (Figures 7, 14, and 22) the next step is to determine if their expression in the fat body is necessary for behavior. Also, since the fat body is a secretory tissue (reviewed in Schlegel and Stainier 2007), it is possible that these courtship-responsive gene products are secreted into the hemolymph, or mediate the secretion of other factors, and function in other reproductively important tissues such as the gonads or nervous system. If we can determine the localization of these behaviorally-responsive gene products we will gain insight into fat body signaling and its role in reproduction.

To better understand how adipose signaling affects behavior, we are studying *female-specific independent of transformer* (*fit*). Not only is *fit* expressed in the fat body (Figures 14 and 15), but it is up regulated by courtship or courtship followed by mating (Figure 14; Tables 3 and 7). This implies that *fit* expression is tied to both courtship sensory cues and mating-induced physiological changes; sensory cues result in rapid (5 or 20 min) changes in *fit* levels but by themselves do not sustain these increases in *fit* (at least 2 hrs later), but post-mating changes result in pro-longed *fit* increases.

Though *fit* does not appear to regulate male-female courtship (Figure 16),

deletion of *fit* results in increased male-male courtship (Figure 17). Previous work studying male-male courtship has typically focused on mutant-mutant interactions without considering that male-male courtship is a binary event (Appendix A). Though mutant-mutant interactions will answer the over-arching question of whether or not a mutation regulates male-male courtship, it does not separate whether the presence of courtship is due to the inability to process sensory information correctly or the presentation of inappropriate courtship cues. These two problems are also not mutually exclusive. In theory, mutations in a gene could affect both processes, though only *fru* and *dsx* have been demonstrated to affect both processes (Appendix A). Examining combinations of *fit* mutant males and control (*fit/+* or *CS*) males reveals that *fit* is involved in both the perception and presentation of sensory cues. We are also curious about the specificity of *fit* in interpreting sensory information. Is *fit* needed for processing the inhibitory cues emittted from mated females or heterospecific flies?

To start to understand how *fit* can affect both processes and whether these processes are linked, we can examine the tissues requiring *fit* to repress either aspect of male-male courtship. The perception or bisexual phenotype would most likely be a nervous system defect. However, reduced fat body expression of *fit* resulted in bisexual courtship. This implies that adipose signaling may alter neural processes. We have yet to confirm Fit's expression in the brain or peripheral sensory organs and are currently evaluating how decreased *fit* expression pan-neuronally affects male behavior. However, if we consider that adipose tissue is linked to the oenocytes in regulating cuticular hydrocarbon synthesis, it is likely that the fat body expression of Fit maintains the

proper pheromonal profiles in males and that reduced *fit* expression may result in males that have increased aphrodisiac hydrocarbons such as 7-pentacosene (7-P) (Antony *et al.* 1985), smell like females, smell like immature males or females, or do not express the inhibitory male pheromones. Generating pheromonal profiles for *fit* mutant males compared to controls will offer more insight.

However, when *fit* expression is reduced in the fat body, including post-developmental reduction, mutant males exhibit both male-male courtship phenotypes (Figures 20 and 21). This implies that adipose signaling is mediating multiple processes. *fit* is likely secreted, since Fit protein is expressed in eggs despite the transcript not being present (Nakahara *et al.* 2005), has a predicted signal sequence, and is expressed in the secretory adipose tissue (Figures 14 and 15). Testing fly hemolymph for the presence of Fit will better answer this question but is a technically challenging task. However, if Fit is secreted as we suspect, or at least involved in the secretion of other factors, it is possible for Fit to affect both the nervous system and the oenocytes or other tissues involved in male courtship behavior.

Behavioral analysis of candidate genes reveal novel functions in male behavior

We hypothesized that courtship-responsive genes would likely regulate male behavior. Therefore, we tested P-element insertions located upstream or within many of the 20 min courtship-responsive genes or the 2 hr courtship and mating-responsive genes for behavioral defects. From this initial analysis, mutants for 2 candidate genes showed decreases in courtship activity.

The courtship-responsive *egh* was required for robust male courtship. Mutations in *egh* resulted in decreased CI values (Figure 8) and further analysis shows that *egh* was required post-developmentally in Apterous neurons for male courtship behavior (Figures 12 and 13); however, we do not know if *egh* expression only in the adult is sufficient for male courtship. *egh* functions in female Apterous neurons comprise a large portion of the CNS, and it is possible that males are utilizing a different set of Ap neurons for courtship behavior from those used in females to regulate the Sex-peptide response. However, there are 2 likely possibilities for the same set of neurons regulating sex-specific behaviors: (1) Egh is interacting with sex-specific factors in the Apterous neurons or (2) the Apterous neurons have sex-specific connections to other neurons creating sexually dimorphic circuitry to regulate two separate behaviors. *fru* establishes a sex-specific neural circuit required for male courtship behavior; however, decreased *egh* expression in *fru* neurons did not affect male courtship behavior. It is likely that *egh* may not be expressed in *fru*-expressing neurons but may affect *fru* neuron function indirectly.

Mutations in the mating-responsive *Jhe* and the functionally-related *clt* reduced CI values (Figure 23) and increased mating latencies (Figure 24) without affecting locomotor activity. *Jhe* levels are undetectable in the head or fat body and thus made further analyses difficult. We sought to reduce *Jhe* levels in the fat body and measure CI values but did not see a reduction in CIs. This may be because *Jhe* is not required in adipose tissue for courtship or because *Jhe* was not reduced by the RNAi mechanism. We cannot rule out the latter possibility. We anticipated that our behaviorally-responsive

genes would be involved in subsequent courtship or mating encounters to make the male a more efficient courter/mater. Indeed, mutations in *Jhe* or *clt* resulted in decreased mating success over 3 mating trials (Figure 25). Another avenue of study is the link between these Juvenile Hormone esterase phenotypes, Juvenile Hormone (JH), and reproduction.

A novel approach to identifying sex-determination target genes

We hypothesized that we could identify other target genes of the somatic sex-determination hierarchy by identifying courtship- and mating-responsive patterns of gene expression. This genetic pathway is the major regulator of Drosophila sexually dimorphic development and is required developmentally and post-developmentally to regulate behavior. We predicted that some candidate genes would be members of this regulatory pathway. Indeed, microarray analysis of sex-determination genes reveals that several of our candidate genes regulated by *tra, fru* or *dsx. CG16772* and *fit* are downstream of *tra*; *CG9377, CG16898, Prx2540-2* are regulated by *fru*, and *dsx* regulates *CG9837* (Goldman and Arbeitman 2007).

Impacts on behavioral genetics

The work presented here is the first to demonstrate that gene expression changes occur in Drosophila males during the performance of reproductive behaviors. We have identified candidate loci whose gene expression levels are modified by courtship or mating experience (Tables 1-4 and 7-8). Several of these behaviorally-responsive genes

are SDH target genes, verifying a novel approach to better understand the genetic components regulating sex-specific developmental and behavioral processes.

In analyzing mutations in these courtship- or courtship and mating-responsive loci, we identified 2 genes with novel functions in modulating behavior. The courtship-responsive *egh* gene, already known to affect development and female behavior, is also required in adult male neurons to modulate male courtship behavior (Figures 10, 12, and 13). Many candidate loci are enriched in the male fat body (Figures 7, 14, 15, and 22; Tables 6 and 10), a tissue recently discovered to affect behavior (Dauwalder *et al.* 2002; Fujii and Amrein 2002; Lazareva *et al.* 2007; Fujii *et al.* 2008; Benito *et al.* 2010; reviewed in Dauwalder 2008). One fat body-expressed gene, *fit*, is also a target gene of the SDH. We have provided the first behavioral analyses of *fit*, which indicate that *fit* modulates both aspects of male-male courtship (bisexual courtship and courtship elicitation) (Figures 17 and 18). *fit* is needed in adult adipose tissue to repress courtship of males and courtship from males (Figures 20-21). As we determine *fit's* role in modulating both aspects of male-male courtship, we will better understand how signaling in the non-neuronal adipose tissue can impact neuronal signaling and behavior.

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APPENDIX A

RESULTS FROM A FlyBase QUERY OF COURTSHIP DEFECTIVE MUTANTS

As I uncovered the *fit* phenotypes (courtship towards or from males), I wanted to determine what other mutants show either phenotype. To generate this comprehensive list of Drosophila courtship defective mutants I first performed a literature search with keyword combinations with Drosophila, courtship, mutant, defective, or male-male courtship, but found this to be a rather tedious and time consuming approach. A more efficient method I took was to utilize the QueryBuilder program found through FlyBase's website at http://flybase.org.

QueryBuilder allows a user to select the query terms from all of FlyBase's variables (e.g. gene, allele, gene ontology) and to define the search parameters for that variable. I searched for alleles that were of the phenotypic class courtship defective. This criterion extracted all alleles (and the corresponding genes) that have been noted as defective in male-female or male-male courtship. The HTML table was exported with the Batch Download HitList Conversion algorithm to generate a tab-separated table of the query which includes the gene symbol, gene name, phenotype summary, and references for further analysis.

I supplemented the FlyBase table with literature searches focusing on male-male courtship mutants. One question to address was whether or not the bisexual or elicitation phenotypes were addressed separately. Unfortunately, most researchers did not parse these phenotypes; mutant males direct courtship towards mutant males (Table A1).

Table A1. Drosophila male-male courtship mutants. Results from a FlyBase query for courtship defective flies. Disruption of dopamine/pale, *dsx,* or *fru* result in both the bisexual & elicitation phenotypes. WT=wild type.

Gene	Symbol	Phenotype	Reference
Male-male courtship mutants			
Dopamine		Increased dopamine causes males to court other males Decreased dopamine results in males eliciting courtship	Liu *et al.* 2008 J Neurosci 21:5539-46 Liu *et al.* 2009 PLoS One 4:e4574
Octopamine		males w/ no OCT or w/ low OCT levels do not adapt to changing sensory cues & court both males & females.	Certel *et al.* 2007 PNAS 104:4706-11
Oenocyte null males		WT males attempted to copulate w/ oe^- males	Billeter *et al.* 2009 Nature 461:987-U250
	CheB42	Males homozygous for $CheB42a^{Î”5\text{-}68}$ display normal levels of overall courtship. The typical climbing response to mechanosensory detection of gravity, stimulation of food intake by gustatory detection of sucrose is not affected in $CheB42a^{Î”5\text{-}68}$ homozygous males. $CheB42a^{Î”5\text{-}68}$ mutant males perform an average of eight attempted copulations in a 10 min observation period, whereas control males perform only three. This increased number results from a faster progression from initiation of courtship behavior to the first attempted copulation, as well as more frequent subsequent attempts. $CheB42a^{Î”5\text{-}68}$ males are not different from controls in the timing or frequency of earlier steps in the courtship sequence (lag to courtship initiation, tracking & following of the female, tapping, & wing vibration). $CheB42a^{Î”5\text{-}68}$ homozygous males spend the same amount of time courting females as WT controls. $CheB42a^{Î”5\text{-}68}$ has no effect on several other behaviors unrelated to courtship; such behaviors include preening, walking, geotaxis, & gustatory response to sugars. The transgene *CheB42a* specifically targets male gustatory sensillae & alters the perception of male inhibitory pheromones which leads to frequent male–male interactions.	Svetec *et al.* 2005 Genetical Res 85:183-93
defective in the avoidance of repellents	*dare*	Mutants also show male-male courtship behavior. No mention of elicitation	Freeman *et al.* 1999 Development 126: 4591--4602
desaturase1	*desat1*	In red light, mutant males could not discriminate the sex of control flies, Under red light conditions, in which flies are effectively blind (Boll & Noll 2002), CS males indiscriminately courted male & female *desatl* mutants (Fig, 3a(C); p=n.s.), whereas they clearly preferred courting female flies when presented w/ male & female control flies (Fig. 3a(A); $p<0.0001$).	Marcillac *et al.* 2005 P Royal Soc B-Biological Sciences 272:303-9

Table A1. Continued

Gene	Symbol	Phenotype	Reference
dissatisfaction	*dsf*	Homozygous females fail to lay eggs voluntarily or under CO_2 anaesthesia. Eggs mature normally in the ovary & pass through the oviducts into the uterus where they degenerate. Homozygous & hemizygous females show significantly longer times from the initiation of courtship until copulation relative to WT. This delay results from active resistance by the female, including running about the mating chamber, wing flicking & kicking the male. Females also show resistance during copulation, showing excess activity & actively trying to dislodge males by flicking their wings, bucking & kicking at the males. Homozygous & hemizygous males initiate courtship w/ mature WT virgin females as rapidly as WT males, & actively court females, but they show a substantially delayed time to copulation compared to WT. The males are defective in abdominal curling, making fewer bends that fall into the maximum degree category (180^o) that is sufficient for copulation. This probably accounts for the increase in time to copulation seen for homozygous & hemizygous males. Homozygous & hemizygous males also actively court both mutant & WT mature males even w/ virgin females present. This includes all courtship behaviors up to & including attempted copulation. WT males do not court homozygous or hemizygous dsf^1 males. Homozygous males are substantially delayed in the average time from initiation of courtship until copulation compared to WT flies. Homozygous males court males as well as females & males show pairwise & multiple male courtship. Young mutant males show minimal head-to-head interaction behavior & almost no homosexual courtship. Mutant males aged individually for approximately 2 wks & then grouped together show a high level of head-to-head interactions on the first day after being grouped together. By 5 days after being grouped together, the level of head-to-head interaction is barely above the WT level, & is significantly lower than the level of head-to-head interaction on the first day after being grouped together. "Swarming" behavior is seen on the first day that the mutant males are grouped together, w/ groups of males moving swiftly throughout the test chamber, seeming to chase other mutant males & be chased by them. When the group of moving males encounters an internal boundary (e.g. the surface of the food) the swarm typically does not break up but moves off in another direction. Swarming behavior persists to day 5 after being grouped together, although the behavior occurs less frequently than on the first day after being grouped together. Males can show head-to-head interactions during swarming behavior, although of shorter duration than head-to-head interactions between a pair of males. Haven't found any evidence that elicitation has been tested	Finley *et al.* 1998 Neuron 21:1363-74 Finley *et al.* 1997 PNAS 94:913-18

Table A1. Continued

Gene	Symbol	Phenotype	Reference
doublesex	*dsx*	XX dsx^1/dsx^1 & $dsx^1/Df(3R)dsx15$ flies do not show any male-specific courtship when paired w/ mature virgin females. 30% of XX & 56% of XY dsx^1 tra^1 double homozygous flies show male-specific courtship when paired w/ mature virgin females. 6% of XY dsx^1/dsx^1 flies & 27% of XY $dsx^1/Df(3R)dsx15$ flies show male-specific courtship when paired w/ mature virgin females. XY $dsx^1/Df(3R)dsx15$ flies have a reduced courtship index (compared to control males) when paired w/ immature male flies. XY males show courtship sluggishness when compared to WT siblings. Aging does not improve the courtship performance. Song pulses are similar to those of dsx^+ males, though number & duration of song bouts are much reduced. No sine-song bouts whatsoever are generated by dsx mutants. In elicitation & rejection tests, dsx haplo-X mutants demonstrate an attractiveness that cannot be explained by general enfeeblement, such as inability to reject courtship advances. dsx^1/dsx^{43} females have a pheromone profile that resembles, to a first approximation, that of WT males. These males court less frequently & less aggressively than $dsx^1/+$ controls, & when they do court it is not sustained for long periods of time. These pseudofemales show little interest in females & perform only early mating behaviors (orientation, tapping, wing extension & vibration). Unlike WT females they continue to move around the chamber during copulation & flick their wings in an apparent attempt to dislodge the male. XY ; dsx^1 males show "normal" levels of head-to-head interactions compared to WT males. Homozygous males form short inter-male chains on day 5 after being grouped together, w/ a maximum of 3 to 4 mutant flies being involved in each chain. Males expressing DsxF are courted by other males.	Waterbury *et al.* 1999 Genetics 152:1653-67 Villella & Hall 1996 Genetics 143:331-344
Ecdysone Receptor	*EcR*	Reduction of EcR-A levels in fru P1-expressing neurons of males caused a significant increase in male-male courtship activity Ganter *et al.* - EcR males do not elicit courtship from WT males but do court WT males	Ganter *et al.* 2007 Behav Gen 37:507-12 Dalton *et al.* 2009 Curr Biol 19:1447-52

Table A1. Continued

Gene	Symbol	Phenotype	Reference
fruitless	*fru*	Homozygous males show chain behavior. fru^{1}/fru^{0-1} males show chain behavior, although not as intensely as either fru^{1} or fru^{0-1} homozygotes, but are essentially normal in fertility. Failure to curl abdomen in attempted copulation. Male heterozygotes w/ In(3R)fru formed courtship chains but were fully fertile & had an abnormal MOL. Males are more stimulated to court females than fru^{3} or fru^{4}. All males that exhibit any courtship do exhibit tapping behavior (tapping of the female w/ the forelegs). Males, when presented w/ both sexes simultaneously, will show a courtship bias toward females. Mutant females are courted by WT males at normal levels. Male-female courtship as measured by wing extension index (WEI) is almost completely abolished in fru^{0-1}/fru^{w9}, fru^{0-1}/fru^{w12}, $fru^{0-1}/Df(3R)fru^{w24}$ or fru^{0-1}/fru^{w27} males. fru^{0-1}/fru^{w9}, fru^{0-1}/fru^{w12} or fru^{0-1}/fru^{4-40} males show substantial chaining. fru^{0-1}/fru^{0-1} & fru^{0-1}/fru^{1} males show longer than normal mating-initiation latencies compared to heterozygous controls when mated to a single virgin WT female. The mating duration is not significantly different from WT. The density of varicosities of the sAbg neurons which are associated w/ the reproductive organs is nearly normal in fru^{0-1} males.	Villella *et al.* 2005 PNAS 102:16550-57 Demir and Dickson 2005 Cell 121:785-94 Manoli *et al.* 2005 Nature 436:395-400 Kimura *et al.* 2005 Nature 438:229-33 Ito *et al.* 1996 PNAS 93:9687-92 Ryner *et al.* 1996 Cell 87:1079-89
fruitless stimulation factor	*fsf*	Homozygous males stimulate WT males to court them even when the mutant males are ether anaesthetised or have their head & thoraces removed. The level of courtship elicited by homozygous males is significantly higher than that elicited by WT males. Heterozygous males stimulate an intermediate level of courtship by WT males. Haven't found data for male discernment	Gailey & Hall 1989 Genetics 121:773-85
garnet	*g*	Mutant flies show disturbed orientation behavior & walking speed in a behavioral assay. g^{1} mutant male flies show a greater degree of male-male courtship than WT flies. Elicitation was not measured	Lloyd *et al.* 2002 Genome 45:296-312
genderblind	*gb*	Males expressing $gb^{dsRNA.Scer\UAS}$ under the control of $Scer\GAL4^{Tub84B.PL}$ show a significant amount of homosexual behavior. Male flies carrying $gb^{KG07905}$ show frequent homosexual interactions, including singing to other males, genital licking & attempted copulation. $gb^{KG07905}$ males presented simultaneously w/ a WT passive (decapitated) male & a WT passive (decapitated) virgin female choose to court the male & female w/ equal intensity & probability (in contrast to WT males which always choose to court females). This phenotype is seen both when group & single-pair courtship assays are carried out. $gb^{KG07905}$ males show much higher homosexual courtship under dim red light conditions (in which the flies are virtually blind) than control males under the same conditions. $gb^{KG07905}$ males show abnormally high courtship levels to mated WT females. Grosjean *et al.* does not deal w/ any elicitation by gb males	Grosjean *et al.* 2008 Nat Neuro 11:54-61
Gustatory Receptor 32a	*Gr32a*	Males w/ a mutated Gustatory receptor 32a gene (Gr32a) show high courtship toward males & mated females. Does not address elicitation	Miyamoto & Amrein 2008 Nat Neuro 11:847-6

Table A1. Continued

Gene	Symbol	Phenotype	Reference
Gustatory Receptor 33a	*Gr33a*	We found that the $Gr33a^{1}$ male flies displayed increased courtship toward passive, decapitated males (Figure 4A). This phenotype was rescued by the $Gr33a^{+}$ transgene (Figure 4A). In contrast, courtship of $Gr33a^{1}$ males to decapitated or normal females was not different significantly from the $Gr33a^{+}$ males (Figures 4B–4D). Did not address elicitation	Moon *et al.* 2009 Curr Biol 19:1623-7
Juvenile hormone esterase binding protein	*DmP29*	Overexpression resulted in male-male courtship behavior. Doesn't look at elicitation	Liu *et al.* 2008 Gen & Comp Endocrin 156:164-72
Odorant receptor 67d	*Or67d*	Mutant males that lack Or67d inappropriately court other males, whereas mutant females are less receptive to courting males. $Or67d^{GAL4-1}$ males paired w/ WT males show a significantly higher male-male courtship index than that of control males paired w/ WT males. $Or67d^{GAL4-1}$ females mated to WT males show a higher copulation latency than control females mated to WT males. The male pheromone cVA (Z-11-octadecenyl acetate) elicits a rapid & robust firing response in the T1 trichoid sensilla of control $Or67d^{+1}$ flies but not in those of $Or67d^{GAL4-1}$ flies. Male & female $Or67d^{GAL4-1}$ flies do not produce a detectable electroretinogram response to cVA (in contrast to controls), but they show a normal electroretinogram response to ethanol. When paired w/ virgin females, $Or67d^{GAL4-1}$ males court at levels comparable to those of control males. However, when $Or67d^{GAL4-1}$ males are paired w/ WT males, they show a roughly threefold higher courtship activity than control males paired w/ WT males. $Or67d^{GAL4-1}$ females mated to WT males show a higher latency to copulation than control females mated to WT males. Virgin $Or67d^{GAL4-1}$ females are courted as vigorously as control females when paired w/ WT males. Application of the male pheromone cVA (Z-11-octadecenyl acetate) to the abdomens of virgin females suppresses courtship by control males but not by $Or67d^{GAL4-1}$ males in a single-pair courtship assay. Reference does not address elicitation	Kurtovic *et al.* 2007 Nature 446:542-6
pale	*ple*	ple^{ts1} males raised at 31°C have significant lower level of dopamine as compared w/ sibling males raised at 25°C. Homozygous ple^{ts1} males raised at the restrictive temperature (31°C) induce homosexual courtship behavior in ple^{ts1} mutant as well as WT males.	Pendleton *et al.* 2002 Behav Genet 32:89-94 Liu *et al.* 2009 PLoS ONE 4:e4574
prospero	*pros*	Heterozygous males show abnormal courtship behavior; they actively court both virgin females & mature males, courting both intact & decapitated flies. The courtship index & attempts at copulation are higher towards females than males. Heterozygous females do not differ for sexual receptivity or locomotor activity when compared to control flies. The courtship index of heterozygous males towards decapitated Canton-S males is significantly higher than that of WT males towards decapitated Canton-S males. No test of elicitation	Grosjean *et al.* 2007 Behavior Genet 37:575-84

Table A1. Continued

Gene	Symbol	Phenotype	Reference
quick-to-court	*qtc*	Sexually mature qtc^1 males perform much more courtship in response to each other than do control Canton-S-5 (CS) males; mature qtc^1 males have a male-male courtship index (CI) of 42 +/- 5 (indicating that courtship behaviors are observed on average during 42% of a 10 min observation period), whereas CS males have a CI of only 4 +/- 1. qtc^1 males' courtship of each other is qualitatively different from that of CS controls. In all pairs of CS males observed, one or both males perform at least one of the "early" behaviors (orientation, tapping & following), but in only half of the pairs observed do one or both males vibrate their wings to produce a courtship song & none of the males lick or attempt copulation. In contrast, in all pairs of qtc^1 males observed, one or both males lick the other male's genitalia & one male attempts to copulate w/ the other male. qtc^1 males do not show chaining behavior, & like CS males, all of the qtc^1 males run away from the males that court them (indicating that the response to male courtship is normal in qtc^1 males). qtc^1 males perform high levels of male-male courtship in response to CS males, while CS males perform little or no courtship in response to qtc^1 males. In qtc^1/CS pairs in which both males perform at least some courtship, the qtc^1 male initiates courtship first. qtc^1 males initiate courtship of CS virgin females quicker than do control CS males. They perform normal levels of courtship towards the females during the observation period: CI is 75 +/- 3 for qtc^1 males paired w/ CS females compared to 78 +/- 3 for CS males paired w/ CS females. The mutant males perform "advanced" courtship behaviors towards the females; all show wing vibration & most show licking of the females' genitalia & curling of the abdomen to attempt copulation. The copulation latencies of qtc^1 males are not significantly different from those of CS males. qtc^1 females elicit as much courtship from CS males as do CS females. Immature qtc^1 males elicit the same level of courtship from CS males as do immature CS males. qtc^1 females are as likely to mate w/ CS males as are CS females, & the mutant females' copulation latencies are normal. Sensory function of qtc^1 flies appears normal (in limited testing of visual & olfactory physiology by electroretinogram & electroantennogram recordings & tests of adult & larval olfactory behavior). No general behavioral defects in activities such as walking or grooming are seen.	Gaines *et al.* 2000 Genetics 154:1627-37
sluggish B	*slgB*	Isolated w/ regard to poor response to light in fast phototaxis tests; both sexes are sluggish in this regard. Males in this strain do not mate w/ females in darkness; in the light, males court & mate w/ females (40-50%, w/in 10 m) & other males w/ equal vigor; the latter behavior includes formations of chains & rings of intermale courters, as well as pseudo-copulation attempts; courtees in these circumstances do not exhibit wing-flick repelling responses characteristic of WT males. Spectral sensitivity studies of the light-elicited courtship activities showed 420-515 nm to be effective (thus orange/red range ineffective); yellow light was perhaps the most stimulatory; quick turn-ons & turn-offs of intermale courtships could be effected by intermittent exposures to yellow & red light, respectively.	Sharma 1977 Experentia

Table A1. Continued

Gene	Symbol	Phenotype	Reference
transformer	*tra*	100% of XX & 86% of XY tra^{4}/Df(3L)st-j7 flies show male-specific courtship when paired w/ mature virgin females. Pseudofemales are placed in individual chambers w/ another male or female they show little interest in courting. When they do court they do not discriminate between males & females & only very early courtship routines, such as orientation, tapping & brief wing vibration are seen. These pseudofemales elicit high levels of courtship from WT males & allow themselves to be mated. The pheromone profile of XY flies carrying $tra^{F.Hsp83}$ resembles that of WT females. Males expressing $tra^{Scer\UAS.cFa}$ under the control of $Scer\GAL4^{pros\text{-}V1}$ show very little courtship towards females & males, for both intact & decapitated target flies. These males rarely attempt to copulate w/ target flies of either sex. Females expressing $tra^{Scer\UAS.cFa}$ under the control of $Scer\GAL4^{pros\text{-}V1}$ are not significantly different from control females w/ respect to sexual receptivity. Transformed males carrying $Scer\GAL4^{7B}$, $Scer\GAL4^{30B}$ or $Scer\GAL4^{53B}$ show a homosexual or bisexual attraction to WT males. Expression of tra in $Scer\GAL4^{c123a}$, $Scer\GAL4^{Tab2\text{-}201Y}$ & $Scer\GAL4^{c739}$ males causes nondiscriminatory courtship. Male courtship can vary from male heterosexual behavior to some bisexual behavior. $tra^{Scer\UAS.cFa}$; $Scer\GAL4^{oeA}$, $tra^{Scer\UAS.cFa}$; $Scer\GAL4^{oeB}$ & $tra^{Scer\UAS.cFa}$; $Scer\GAL4^{oeC}$ males secrete a mix of sex pheromones more similar to WT females than males & induce similar levels of courtship behavior in WT males as WT females do. $tra^{Scer\UAS.cFa}$; $Scer\GAL4^{oeD}$ or $tra^{Scer\UAS.cFa}$; $Scer\GAL4^{oeE}$ males also secrete a feminised mix of sex pheromones & induce significantly higher levels of courtship behavior in WT males than other WT males, but not as much as WT females do. In contrast, the mix of sex pheromones secreted by $tra^{Scer\UAS.cFa}$; $Scer\GAL4^{noeF}$ or $tra^{Scer\UAS.cFa}$; $Scer\GAL4^{noe.G}$ males is similar to that secreted by WT males. These males do not induce significantly higher levels of courtship behavior in WT males than other WT males do. Expression of $tra^{Scer\UAS.cFa}$ driven by $Scer\GAL4^{hs.PB}$ reduces the amount of cuticular hydrocarbons by about 93% & renders both known & suspected sex pheromones undetectable in female flies. Over 95% of control males court decapitated $tra^{Scer\UAS.cFa}$,$Scer\ GAL4^{hs.PB}$ females, showing the full range of courtship, though the Courtship Index (CI) of these females is less than 2/3 of that of WT females. No reduction of CI is seen w/ intact living females of the same genotype. In heterospecific courtship tests w/ heatshocked $tra^{Scer\UAS.cFa}$,$Scer\ GAL4^{hs.PB}$ D.melanogaster females & D.sechellia, D.simulans, & D.mauritiana males, relatively high levels of courtship are seen - the CIs observed are consistently significantly higher than w/ non-heatshocked females. When heatshocked $tra^{Scer\UAS.cFa}$,$Scer\ GAL4^{hs.PB}$ D.melanogaster females are treated w/ D.melanogaster cuticular hydrocarbons, CIs are reduced to WT levels. When heatshocked $tra^{Scer\UAS.cFa}$,$Scer\ GAL4^{hs.PB}$ D.melanogaster females are treated w/ D.simulans cuticular hydrocarbons, CIs w/ WT D.melanogaster & D.sechellia males are reduced, CIs w/ WT D.simulans, & D.mauritiana males are increased. In single-pair cross interspecific mating tests between $tra^{Scer\UAS.cFa}$,$Scer\ GAL4^{hs.PB}$ D.melanogaster females & WT D.sechellia, D.simulans, & D.mauritiana males, mating frequencies are significantly increased from the 0% seen w/ WT females. The courtship index of Canton-S male flies in response to XY flies expressing $tra^{Scer\UAS.cFa}$ under the control of a number of Scer\GAL4 lines ($Scer\GAL4^{53B}$, $Scer\GAL4^{10B}$, $Scer\GAL4^{32B}$, $Scer\GAL4^{30B}$ or $Scer\GAL4^{6J3}$) is higher than that of Canton S male flies in response to control XY males.	Taylor *et al.* 1994 Dev Genet 15:275-96 Waterbury *et al.* 1999 Genetics 152:1653-67 Ferveur *et al.* 1997 Science

Table A1. Continued

Gene	Symbol	Phenotype	Reference
Vesicular glutamate transporter	*VGlut*	Males expressing VGlut$^{Scer\UAS.cDa}$ under the control of Scer\GAL4$^{Î±Tub84B.PL}$ show high levels of homosexual courtship, which includes all aspects of sexual behavior (including singing, genital licking & attempted copulation). Occasionally, the males attempt copulation w/ inappropriate body regions, such as the head. Males expressing VGlut$^{Scer\UAS.cDa}$ under the control of Scer\GAL4^{NP0225} show a significant amount of homosexual behavior, although less than seen when the Scer\GAL4$^{Î±Tub84B.PL}$ driver is used. No test of elicitation	Grosjean *et al.* 2008 Nat Neurosci 11:54-61
white	*w*	Transformant heat shocked males exhibit homosexual courtship behavior. Male-male courtship occurs between flies following ectopic expression of w^{+mC}. Male-male courtship behavior increases w/ age, flies 1 wk old or older show high levels of courtship compared to one day posteclosion males. Olfactory, visual & gustatory responses are not affected by heat shock. Surgical ablation of the antenna &/or maxillary palps, or the wings has no effect on male-male courtship. Under red light male-male courtship is substantially reduced, restoration of white light increases courtship. For all classes of surgically treated flies red light diminishes courting activity. Male-male courtship is not due simply to an absence of rejection behavior (wing flicking) as heat shocked males will court a non-w^{+mC} male exhibiting rejection behavior. Also the courting is not due to increased sexual attractance as non-w^{+mC} males will not court heat shocked w^{+mC} males.	Hing *et al.* 1996 J Neurobio 30:454-64 Zhang & Odenwald 1995 92:5525-9 Anaka *et al.* 2008 J Neurogen 22:243-76 An *et al.* 2000 J Neurogen 14:227
Male-female courtship mutants			
amnesiac	*amn*	Unlike WT males, amn^{28A} homozygous males that have undergone courtship conditioning (kept in the presence of a female for 7 hrs) do not spend significantly less time engaged in courtship behavior when placed w/ a female 5 days after conditioning than non-conditioned males of the same genotype. Unlike WT males amn^{28A} homozygous males conditioned to the presence of a female for 30 min do not spend significantly less time engaged in courtship behavior when returned to the presence of a female 1 hour later than non-conditioned males. Mutant males learn courtship suppression normally if tested immediately after training w/ a mature decapitated virgin & do not avoid mature virgins after training w/ an immature female. Introducing a delay between training & w/ a mature virgin & testing uncovers a more rapid decay of memory in these mutants.	
apterous	*ap*	Female sterile w/ underdeveloped ovaries; 6nurse cell nuclei become pycnotic after stage 7 & stage-8 oocytes are the most advanced. Males show immature sexual behavior & are sterile, but testes appear normal w/ motile sperm. Mutant males spend less time courting & are less likely to perform some of the courtship behaviors than age-matched controls. Abnormalities are an indirect effect of the mutation which causes partial paralysis, lack of coordination & sluggishness. Only 13% female receptivity to mature male ap^{+} flies. Female flies have a high courtship intensity, but low percentage mating: this is due to low receptivity not low 'sex appeal'. Homozygous ap^{4} males are behaviorally sterile but have fertile gametes. Homozygous females are sterile; the ovaries are poorly developed, fragile & contain no vitellogenic egg chambers.	Ringo *et al.* 1992 22:469-87

Table A1. Continued

Gene	Symbol	Phenotype	Reference
ADP ribosylation factor 51F	*Arf51F*	Mutant females show reduced fertility. Mutant males are completely sterile. Mutant spermatids have a "four-wheel-drive" phenotype, indicating a cytokinesis defect during spermatocyte meiosis; more than 90% of spermatids have more than one nucleus.	
ariadne	*ari-1*	They also fail to perform any of the sexual routines (wing song, dance, licking & mounting) in front of WT females.	
	ato	Oenocytes do not form in homozygous embryos, where the formation of the primary sensory organ precursors (SOPs) is compromised in most segments. In segments where remnant SOPs do develop, oenocytes can develop. Mutant males demonstrate vigorous courtship including courtship songs. 66% of mutant males use both of their wings simultaneously during courtship song production (in contrast to WT males which vibrate only one wing at a time). Relative amplitude of the sine song & pulse number is higher than normal in mutant males. Pulse duration is significantly longer than WT.	
bab2	*bab2*	Expression driven by Scer\GAL4$^{pnr\text{-}MD237}$ in males causes loss of both male specific & non-sex-specific striped abdominal pigmentation.	
Btk family kinase at 29A	*Btk29A*	The apodeme at the base of the penis apparatus is split distally into 2. Flies exhibit a highly variable copulatory span & repetitive copulation in mated pairs often occurs soon after separation. Copulation involving mutant Btk29A$^{fic\text{-}P}$ males often terminates shortly after initiation. Mating behavior of Btk29A$^{fic\text{-}P}$ females is normal. Mutant males exhibit prominent atrophy of the posterior ejaculatory duct.	
beethoven	*btv*	Audiograms demonstrate mutants show a reduced response at all sound intensity. Mutants respond abnormally in an olfactory behavior test. 6/20 males successfully copulate w/ females w/in 30 min. Males are fertile w/ WT sperm motility. Mutants show reduction in sound-evoked courtship behavior. Mutant males demonstrate vigorous courtship including courtship songs. 50% of mutant males use both of their wings simultaneously during courtship song production (in contrast to WT males which vibrate only one wing at a time). Mutant males fully twist the vibrating wing so that the ventral surface is directed forward (90^{o} angle of attack), whereas WT males only twist the vibrating wing so the trailing edge is slightly lower. Pulse duration is significantly longer than WT in mutant male songs & the carrier frequency of the sine song is significantly higher than normal. Relative amplitude of the sine song is higher than normal in mutant males.	
c601	*c601*	c601 virgin females show elevated ovulation; 20% of the virgin females show ovulation (compared to 0% of virgin Oregon-R females). The flies show no obvious defects in viability, fertility, morphology or locomotor activity. The courtship index observed when c601 virgin females are mated to WT males is lower than that seen when WT virgin females are mated to WT males, but is higher than that seen when WT mated females are mated to WT males. c601 mutant virgin females show ovipositor extrusion towards courting WT males (this extrusion behavior is normally observed in mated WT females but not in virgin WT females).	

Table A1. Continued

Gene	Symbol	Phenotype	Reference
c625	*c625*	c625 virgin females show elevated ovulation; 46% of the virgin females show ovulation (compared to 0% of virgin Oregon-R females). The flies show no obvious defects in viability, fertility, morphology or locomotor activity. The courtship index observed when c625 virgin females are mated to WT males is lower than that seen when WT virgin females are mated to WT males, but is higher than that seen when WT mated females are mated to WT males. c625 mutant virgin females show ovipositor extrusion towards courting WT males (this extrusion behavior is normally observed in mated WT females but not in virgin WT females).	
cacophony	*cac*	Males court abnormally w/ poor mating success & aberrant courtship song, which includes pulses of tone that are polycyclic, rather than monocyclic or tricyclic, w/in WT pulses & have increased amplitude. Mating success of wingless mutant males is still worse than that of wingless WT males, which is correlated w/ genetic separability of song abnormalities from deficit in mating performance. Female courtship appears to be unaffected by cac; but general locomotor activity of males or females is subnormal.	
Calcium/calmodulin-dependent protein kinase	*Caki*	Walking speed & path length of $Caki^{X\text{-}307}/Caki^{X\text{-}313}$ transheterozygotes is considerably reduced, as studied in Buridan's paradigm. Vision seems not to be dramatically altered. $Caki^{X\text{-}307}/Caki^{X\text{-}313}$ double mutants fail to suppress courtship w/ mated females as normal & are defective for courtship habituation. Associative memory, is normal in mutants.	
Calmodulin	*Cam*	The initial stages of male courtship are normal, but the rescued males cannot bend their abdomens sufficiently to achieve penetration.	
Calcium/calmodulin-dependent protein kinase II	*CamKII*	Males expressing $CaMKII^{T287A.Scer\backslash UAS}$ under the control of either $Scer\backslash GAL4^{MJ85b}$ or $Scer\backslash GAL4^{30Y}$ show the same level of courtship training following 1 hour w/ mated females as WT flies.	
celibate	*cel*	Males court females vigorously but rarely attempt to copulate & even less frequently achieve genital contact; females apparently unaffected by the mutation.	
coitus interruptus	*coi*	Males court females w/ slightly reduced vigor & mating success but mate frequently; duration of such copulations average 60% of normal 20 min; high proportion of mutant males also have abnormal sperm, e.g., nonmotile; females apparently unaffected by the mutation.	
courtless	*col*	Very early steps of courtship behavior are disrupted. Spermatogenesis is abnormal. 78% of homozygous males do not court WT virgin females at all & 17% perform only some of the courtship steps. Most of the mutant males that do court (13%) perform only early steps of courtship (orienting, following & wing extension). Only 5% of homozygous males eventually copulate, & these matings give no progeny. The courtship index is 12% for homozygous males, compared to 72% for WT males (breaking down this value for courting & noncourting mutant males gives a C.I. value of 0 & 23% respectively). Homozygous females are fertile when mated to WT males, behave normally & are as receptive to males as are WT females.	Orgad *et al.* 2000 Genetics 155:1267-80

Table A1. Continued

Gene	Symbol	Phenotype	Reference
croaker	*cro*	Male courtship pulse song is clearly different from WT, it is polycyclic. Also the interpulse intervals are statistically longer than WT. The courtship sine song begins w/ a lower amplitude & does not maintain the level of loudness. Mating success of males w/ WT females is low, male courtship is not as vigorous as that of WT males. Mating success of females w/ WT males is also low.	
Cysteine string protein	*Csp*	Homozygous males seem too feeble to show any sign of courtship behavior. The courtship song parameter interpulse interval is normal in heterozygous males at 25^{o}C, but the number of cycles per pulse & amplitude of sound are significantly higher than in WT males at 25^{o}C, & the interpulse frequency is significantly lower than in WT males at 25^{o}C.	
cycle	*cyc*	The pronounced circadian rhythm of close-proximity encounters seen in WT male:female pairs is lost when the male is homozygous for cyc^{01}. cyc^{01} mutant females mated to cyc^{01} mutant males lay significantly less eggs & produce significantly less progeny than WT flies. The percentage of unfertilised eggs from this mating is not significantly greater than for WT. There is no significant increase in the time spent copulating by pairs of cyc^{01} homozygous flies compared to WT pairs. Long term memory of courtship conditioning (reduction in time spent in courtship behavior 5 days after a 7 hour conditioning) is normal in cyc^{0} homozygous males.	
Cyp4d21	*Cyp4d21*	Homozygous mutant males have a normal courtship index, but there is a significant increase in the fraction of homozygous males that do not mate compared to control males. Males expressing $Cyp4d21^{dsRNA.Scer\UAS}$ under the control of Scer\ $GAL4^{Cyp4d21.PF}$ show a significantly reduced courtship index & a significantly reduced mating success compared to control males.	
don giovanni	*dg*	Males not conditioned by courtship of fertilized females, apparently because they fail to elicit the appropriate cues from them; this means, further, that after a dg^{1} male courts a fertilized female, a WT male will not show the usual depressed courtship of this female; yet if the fertilized female is first courted by a WT male, a dg^{1} male will exhibit depressed courtship of her; females fertilized by dg^{1} males do not effectively modify subsequent courtships directed at them by any male type.	
dunce	*dnc*	Unlike WT males, dnc^{1} homozygous males that have undergone courtship conditioning (kept in the presence of a female for 7 hrs) do not spend significantly less time engaged in courtship behavior when placed w/ a female 5 days after conditioning than non-conditioned males of the same genotype. Mutant males fail to show trainer-specific suppression of courtship, decreasing courtship toward a mature virgin after training w/ either mature or immature females. the ability to discriminate between mated & virgin females remain intact.	
ether a go-go	*eag*	When expression is driven by Scer\$GAL4^{MJ286}$ or Scer\ $GAL4^{MJ286}$ the mean courstship latency is decreased, when driven by Scer\ $GAL4^{MJ286}$ the mean courstship latency is increased. Scer\ $GAL4^{MJ286}$ causes increase in mean duration of courtship. When expression is driven by Scer\$GAL4^{7B}$, Scer\$GAL4^{6J3}$, Scer\$GAL4^{53B}$, Scer\$GAL4^{40B}$, Scer\$GAL4^{28A}$, Scer\$GAL4^{c309}$, Scer\$GAL4^{c747}$, Scer\$GAL4^{OK348}$, Scer\$GAL4^{29B}$, Scer\$GAL4^{MJ63}$ or Scer\$GAL4^{MJ146}$ wing extension & vibration duration during courtship are increased.	

Table A1. Continued

Gene	Symbol	Phenotype	Reference
elongase F	*eloF*	Females expressing eloF$^{dsRNA.Scer\UAS}$ under the control of Scer\GAL4^{OK72} show no overall quantitative change in hydrocarbon levels compared to control females, but the hydrocarbon profile is greatly altered; there is a 554% increase in 7,11-dienes of 25 carbons & a 73% decrease in 7,11-dienes of 29 carbons. There is also a small but significant decrease in C27 monoenic & saturated hydrocarbons at the expense of C23. Expression of eloF$^{dsRNA.Scer\UAS}$ under the control of Scer\GAL4^{OK72} in males have no significant effect on hydrocarbon profiles. WT males show a decrease in both the number of copulation attempts & the courtship index & an increase in the copulation latency when mated to females expressing eloF$^{dsRNA.Scer\UAS}$ under the control of Scer\GAL4^{OK72} compared to when WT males are mated to control females. WT males given the choice of mating w/ WT females or females expressing eloF$^{dsRNA.Scer\UAS}$ under the control of Scer\GAL4^{OK72} exclusively mate w/ the WT females.	
Fad2	*Fad2*	Male & female flies expressing Fad2$^{dsRNA.Scer\UAS}$ under the control of Scer\GAL4^{OK72}, Scer\GAL4$^{shn-NP5250}$ or Scer\GAL4$^{Lsp2.PH}$ show no difference in the total amount of hydrocarbons compared to controls. Females expressing Fad2$^{dsRNA.Scer\UAS}$ under the control of Scer\GAL4^{OK72} show a large decrease in 7,11-HD (-83%) & 7,11-ND (-85%) & an increase in 7-monoenes compared to controls. Females expressing Fad2$^{dsRNA.Scer\UAS}$ under the control of Scer\GAL4$^{shn-NP5250}$ show a significant decrease in diene percentages in favour of monoenes compared to controls. Expression of Fad2$^{dsRNA.Scer\UAS}$ under the control of Scer\GAL4$^{Lsp2.PH}$ has no significant effect on the hydrocarbon profile of female flies. Expression of Fad2$^{dsRNA.Scer\UAS}$ under the control of Scer\GAL4^{OK72}, Scer\GAL4$^{shn-NP5250}$ or Scer\GAL4$^{Lsp2.PH}$ has no significant effect on the hydrocarbon profile of male flies. WT males mated to females expressing Fad2$^{dsRNA.Scer\UAS}$ under the control of Scer\GAL4^{OK72} show a decrease in the number of copulation attempts & the courtship index & an increase in copulation latency compared to when WT males are mated to control females. Females expressing Fad2$^{dsRNA.Scer\UAS}$ under the control of Scer\GAL4^{1407} show a 27% increase in linear saturated hydrocarbons compared to controls. The amount of dienes & pheromones is decreased by 98%, while that of monoenes is doubled. WT males show altered courtship behavior towards females expressing Fad2$^{dsRNA.Scer\UAS}$ under the control of Scer\GAL4^{1407} compared towards WT females; there are 12% fewer copulation attempts & 15% less copulation. Courtship, the first copulation attempt & copulation latencies are increased by 65%, 115% & 55% respectively.	
freeze	*fez*	Males do not show any courtship behavior.	
flamenco	*flam*	Mutant males show decreased mating activity. The males show changes in courtship compared to WT; there is a delay in the transition from the orientation stage to the vibration stage.	Suotcheva *et al.* 2003 Russ J Genetics 39:553-8 Romanova *et al.* 2000 Russ J Genetics 36:400-3
flight reduced 393	*flrd393*	Usage of wings by courting males abnormal.	

Table A1. Continued

Gene	Symbol	Phenotype	Reference
flight reduced 397	*flrd397*	Usage of wings by courting males abnormal.	
flight reduced C	*flrdC*	Flight weak; adults seem generally feeble, including poor jumping ability; 10-30% of adults fail to inflate wings normally; even when wings inflated, males seldom extend them fully when courting.	
flight reduced H	*flrdH*	Weak to very weak flight; small amplitude of wing beats in flight; weak jumping ability; decreased viability, flying ability, or both when raised at higher temperatures. Causes abnormalities of wing movements in courtship after rearing at 29^{o}C.	
Fmr1	*Fmr1*	Mutant males spend significantly less time than WT engaged in courtship, & fail to engage in advanced courtship behavior - progressing no further than following/tapping. Animals carrying Fmr1^{I244N} or Fmr1^{I307N} in a homozygous Fmr1^{3} background show a reduced courtship index towards virgin females than that seen in WT males.	
gate	*gat*	Fluctuations of male's courtship song interpulse intervals define a sloppy rhythm or are arrhythmic.	
Gustatory Receptor 68a	*Gr68a*	40% of mutant males did not mate w/ control females; those that did had increased mating latencies	Bray & Amrein 2003 39L1019-29
homer	*homer*	Mutant males show behavioral plasticity deficits & fail to form &/or retain conditioning by the non-receptive mated female in courtship conditioning assays. Mutants do suppress courtship behavior during conditioning but show higher levels of courtship than WT both before & after conditioning.	
	Hsap	Male flies expressing Hsap\PQBP1$^{Scer\UAS.cYa}$ under the control of Scer\GAL4$^{elav.PLu}$ exhibit abnormal courtship behavior approximately 80% of the time, compared to never in control flies.	
	hypo	When homozygous in females, causes reduced mating propensity & extended courtship durations.	
inactive	*iav*	For each age mutant flies are more attractive than WT flies, the attractiveness does decrease w/ age. iav^{1} adults are extremely inactive. Intermediate experience-dependent courtship modification (EDCM) phenotype. In tests of homozygous iav^{1} females (w/ normal males), mating propensity reduced & courtship durations extended, though such females showed normal compositions of cuticular hydrocarbons & were highly attractive to courting males. Mutant males w/ normal females exhibited slightly reduced mating success; mutant males crossed w/ mutant females had very low mating success rate.	

Table A1. Continued

Gene	Symbol	Phenotype	Reference
icebox	*ibx*	Lays eggs. No sperm is stored in hemizygous females. Homozygous & hemizygous mutant flies are fertile except for a few never-mating females. The mating frequency of homozygous females is reduced about 4-fold compared to WT levels, for virgins aged between 1 to 12 days. The largest difference from WT occurs at 3 days of age, when the mating frequency of homozygous females averages 21% versus 88% of WT controls. The changes in receptivity w/ age are similar in homozygous & WT females. The mutation does not appear to be temperature sensitive (in terms of temperature of development). The sex appeal of homozygous females (measured as min of wing vibration directed towards the female by a male in a 5 min test) is indistinguishable from that of WT females. Homozygous females show rejection behavior (such as decamping, wing flicking, kicking, fending & ovipositor extrusion) in response to male courtship more frequently than control females. General locomotor activity of homozygous females, during courtship w/ WT males, is not different from WT. Mutant males do not differ significantly in any component of courtship, except that mutant males give significantly more bouts of abdomen bending than WT males. The mating success of mutant males is not significantly different from that of WT males. The egg-to-adult viability of homozygotes & the progeny of a cross between homozygotes & WT is normal. Ovarian development of homozygous females is normal. Attraction to ethanol & avoidance of 3-octanol is normal in mutant males & females, & photobehavior is normal. Treatment w/ methoprene results in more mating in homozygous females.	O'Dell 1993 Heredity 70:393-9
infertile crescent	*ifc*	Heterozygotes w/ ifc^1 show male sterility & abnormal spermatids.	
inactivation no afterpotential C	*inaC*	Heat shocked males are tested for courtship conditioning. They are placed in the same chamber w/ an unreceptive mated female, males fail to exhibit the effects of conditioning, courtship to the mated females is not suppressed. Despite the failure to show immediate suppression of courtship they do develop normal memory of it. When placed w/ a virgin female they exhibit reduced courtship. Retention of conditioning is seen over 10 min or 2 hrs. This result is comparable to non-heat shocked siblings. Males appear to be forming normal memory retention despite their failure to show any sign of associative learning while it is occurring.	
intersex	*ix*	XY, ix^5/ix^5 males elicit much less courtship than their control siblings. XX, ix^5/ix^5 are almost female-like in courtship behavior.	Acharyya & Chatterjee 2002 Genetical Res 80:7-14 McRobert & Tompkins 1985
Lesbian	*Les*	Females exhibit male-like courtship, including unilateral wing display, directed at other females. Courtship bouts are relatively short. Not all females of the strain show the anomalous behavior.	

Table A1. Continued

Gene	Symbol	Phenotype	Reference
lingerer	*lig*	Homozygous males do not show any generalised behavioral abnormality (such as inactivity, sluggishness or uncoordinated walking). Homozygous males initiate copulation w/ WT females just as often as WT males do, but are frequently unable to release the female's genitalia while mounted on the female's back. The mutant males tend to dismount the females w/out w/drawing their genitalia, & as a result, the male & female often tug at each other, pulling in opposite directions. In many cases, the mutant male & WT female remain stuck for several seconds to tens of min after copulation, until finally managing to separate from each other. 75% of mutant males mated to WT females show this "stuck" phenotype. Mutant females do not show any abnormalities in courtship behavior, although they show only approximately 25% of the fertility of WT females. The mutant males are as active as WT males in performing the early steps of mating behavior (chasing, tapping, singing & licking). After these steps, 73% of the lig^{P1}/lig^{PP1} males attempt copulation w/ WT females, but only 24% of these succeed in copulation. $lig^{P1}/Df(2R)lig^{X4}$, $lig^{P1}/Df(2R)lig^{X13}$ & $lig^{P1}/Df(2R)lig^{X18}$ males show reduced mating success compared to WT.	
Methoprene-tolerant	*Met*	Mutant males court & mate WT females less avidly than controls	Wilson *et al.* 2003 Insect Biochem Mol Biol 33:1167-75
Myb-interacting protein 40	*mip40*	Most homozygous females are completely sterile, while a few give rise to one or two larvae after 7 days of egg laying. Homozygous males are sterile. The mutant testes completely lack mature sperm & development appears to be arrested at the primary spermatocyte stage, prior to the G2-M transition.	
maleless	*mle*	P{HA-DQIH} males have low viability & fertility. Many males hold their wings out from their body.	
Monoplane	*Mpe*	Homozygotes are sterile & heterozygotes are partially sterile. Wings are held out horizontally & at 90^{o} to the body, in a position very similar to that of tx mutants. The wings appear normal in all other aspects, although the flies are unable to perform certain courtship wing movements & are flightless.	
no extended memory	*nemy*	Mutant males show distinct memory deficiency in two courtship conditioning assays. These defects are apparent 0.5 hrs after training. The mutant males show an increased level of locomotor activity unrelated to courtship & spend more time in such an element of courtship as pursuit.	
nerd	*nerd*	Defective sexual behavior in males. Homozygotes exhibit low levels of copulation w/ WT females. Males are affected in their production of predominant cuticular hydrocarbons.	Ferveur & Jallon 1993 Naturwissenschaften 80:474-5
no on or off transient A	*nonA*	Isolated as song mutant. In general short pulse trains (5 pulses or less) of the courtship song are normal in males, but although pulse trains containing more than 5 pulses begin normally, they break into polycyclic, high amplitude pulses. These polycyclic pulses have an amplitude 50-100% higher than WT. Males exhibit longer than normal mating latencies in their courtship of females. Females may be subnormally receptive to males.	Rendahl *et al.* 1992 J Neurosci 12: 390-407

Table A1. Continued

Gene	Symbol	Phenotype	Reference
ovarian tumor	*otu*	Oncogenic allele; 91% of homozygous ovarioles contain tumorous chambers consisting of a mass of small, undifferentiated, mitotically active cells. 85% of hemizygous ovarioles are quiescent. The lifespan of mated homozygous females is significantly shorter than that of unmated homozygous females. Spermatogenesis normal but mating behavior fails because WT females refuse to react to the courtship attempts of mutant males. Effect is not absolute, & depends on allele (there is a strong correlation between male sterility & severity of impairment in the female phenotype) & varies between affecting 6.7% (otu^{14}) & 86.6% (otu^{12}) of mutant males.	
parkin	*park*	Both male & female $park^{unspecified}$ homozygotes are sterile, & males do not exhibit many courtship behaviors.	
period	*per*	Spectral analysis of tone pulse production demonstrated that per^{s} male courtship song is not strongly rhythmic. Females have a preference for WT courtship song over the mutant short song rhythm. Single per^{01} mutant females mated once to single per^{01} mutant males lay significantly less eggs & produce significantly less progeny than WT flies. The percentage of unfertilised eggs from this mating is not significantly greater than for WT. These effects on fertility are significantly reduced if pairs of per^{01} mutant flies are allowed to mate over a period of 4 days. The time spent copulating is significantly increased for per^{01} mutant males mated to WT females, compared to WT males. This effect is seen in both 2 day old & 4 day old males. This increased time in copulation does not appear to be due to difficulty of males disengaging from females. Unlike WT males, per^{01} homozygous males that have undergone courtship conditioning (kept in the presence of a female for 7 or 9 hrs) do not spend significantly less time engaged in courtship behavior when placed w/ a female 5 days after conditioning than non-conditioned males of the same genotype. This phenotype is unaffected if the 7 hour conditioning is performed during nighttime (Zeitgeber time, 12-19). However, 11 hrs courtship conditioning is sufficient to reduce courtship behavior 5 days later in these flies. Courtship conditioning of 30 min is also sufficient to significantly reduce courtship behavior 1 hour after conditioning in per^{01} males.	Roche *et al.* 1998 Behavior Genetics 28:391-4
Pox neuro	*Poxn*	Under daylight conditions, 2/3 of $Poxn^{\hat{I}\text{"}M22\text{-}B5}$ males do not initiate courtship in single choice experiments w/in 15 min under st&ard conditions, while the remaining 1/3 court females very weakly, but do proceed through the entire courting sequence. Although these males do attempt to copulate by bending their abdomen, no copulation has been observed. $Poxn^{\hat{I}\text{"}M22\text{-}B5}$ males show no courtship behavior under red light. $Poxn^{\hat{I}\text{"}M22\text{-}B5}$ males have no penis, although the penis apodeme & protractor muscle are still present.	Boll & Noll 2002 Development 129:5667-81
pickpocket 25	*ppk25*	Mutations in the ppk25 gene reduce or even abolish male courtship response to females in the dark, conditions under which detection of female pheromones is an essential courtship-activating sensory input. ppk25 mutant males that show no response to females in the dark execute all of the normal steps of courtship behavior in the presence of visible light, suggesting that ppk25 is required for activation of courtship behavior by chemosensory perception of female pheromones	Lin *et al.* 2005 PNAS 102:12831-6

Table A1. Continued

Gene	Symbol	Phenotype	Reference
P-element somatic inhibitor	*Psi*	Psi^{v16} males partially rescued by $Psi^{I''AB}$ show courtship defects. The courtship index of these males is 60% (compared to 75% in controls). Also only 30% of mutant males copulate over a 30-min period & these matings yield no progeny.	
phosphatidylserine receptor	*PSR*	Expression of $PSR^{Scer\UAS.cKa}$ under the control of either $Scer\GAL4^{Act5C}$ or $Scer\GAL4^{tub}$ results in males w/ a rotated genitalia defect.	
retained	*retn*	$retn^{z2\text{-}428}/retn^{05096}$ females exhibit male like courtship behaviors. Mutant females follow, tap & appear to sing. Although not as robust as male courtship - following is not as sustained, full wing extension & vibration is not seen, & copulatory bending is weak or absent - these behaviors resemble courtship. The peak penetrance of these phenotypes is 3-4 wks post eclosion, averaging 42 courtship events per 5 min observation period (compared to <3 in controls). male-male & male-female courtship in $retn^{z2\text{-}428}/retn^{05096}$ mutant males is comparable to WT.	
rickets	*rk*	rk^{4} male mates w/ WT female only if wings removed from female.	
raised	*rsd*	Viability & fertility normal. Wings are held upright. Males cannot vibrate their wings to produce the courtship song, as expected males are less successful when courting WT females.	
shaking A	*ShakA*	males show abnormal wing usage in courtship.	
shibire	*shi*	Males containing $shi^{ts.Scer\UAS}$; $Scer\GAL4^{fru\text{-}GAL4}/+$ somatic clones induced using $Scer\FLP1^{ey.PN}$ paired w/ WT virgin females have low courtship index scores at the non-permissive temperature (30°C) compared to $Scer\GAL4^{fru\text{-}GAL4}/+$; $Scer\FLP1^{ey.PN}$ controls.	
	sim2	Of flies w/out external defects, $sim^{J1\text{-}47}/sim^{2}$ males do not perform normal courtship behavior & $sim^{J1\text{-}47}/sim^{2}$ females ignore WT males. Most $sim^{J1\text{-}47}/sim^{2}$ adults only walk in circles & they show abnormal responses in Buridan's paradigm; they show a markedly reduced walking speed, the activity period is usually shorter & they show no measurable influence from visual l&marks on their orientation behavior.	
slowpoke	*slo*	Homozygous males produce very low amplitude courtship songs w/ long interpulse intervals, & low interpulse frequency & cycles per pulse values compared to WT males. Isolated putative pulses, usually monocyclic signals, often occur. Males show many courtship wing extensions w/out actually producing audible sound. The interpulse frequency of heterozygous males is significantly lower than in WT males at 25°C, although other courtship song parameters of heterozygous males are normal.	

Table A1. Continued

Gene	Symbol	Phenotype	Reference
spinster	*spin*	Low mating success: virgin females perform repeated repelling movements that cause abortive mating. Low mating success, females exhibit strong mate-refusal. Receptivity reaches maximum on day 2 (12.5% of WT levels) & declines rapidly thereafter. Only 4% of mutant females that are paired w/ WT males copulate during an assay period of 1 hour (70% of WT females copulate w/ WT males under the same assay conditions). The time spent by the WT male performing unilateral wing vibration during a 10 min observation (the SAPI - sex appeal parameter index) is almost identical for males paired w/ mutant or WT females, indicating that the low mating success of mutant females is not due to reduced attractiveness. Mutant females consistently display a number of rejection responses when paired w/ WT males, including fending, kicking, flicking, curling, punching & decamping, although extrusion is rarely seen. In response to approaching males, the mutant females tend to raise their abdomens while spreading their vaginal plates (this spreading behavior is not seen in either WT virgin or fertilised females & is distinct from extrusion, in which the ovipositor protrudes from the female terminalia). The mutant females often rush towards the courting male, pushing the male's head w/ their forelegs (this aggressive "punching" behavior is rare amongst WT females). $spin^{1}/spin^{10403}$ females show a number of rejection behaviors when paired w/ WT males, including fending, kicking, flicking, curling, punching & decamping, although extrusion is rarely seen. Mutant male flies show no obvious abnormality in their courtship behavior. Homozygous females rarely lay eggs.	Nakano *et al.* 2001 Mol Cell Biol 21:3775-88
sarah	*sra*	Expression of sra^{GS3080} under the control of Scer\GAL4$^{elav\text{-}C155}$ or Scer\GAL4$^{sca\text{-}537.4}$ results in increased ovulation in virgin females compared to control virgin females (the effect is stronger w/ Scer\GAL4$^{elav\text{-}C155}$). W/in 30 min in a mating assay, only 20% of virgin females expressing sra[GS3080] under the control of Scer\GAL4$^{elav\text{-}C155}$ have mated, compared to 82% of WT virgin females. Virgin females expressing sra[GS3080] under the control of Scer\GAL4$^{elav\text{-}C155}$ elicit intermediate courtship index (CI) levels (CI is the period of time w/in the observation period that a male displays any element of courtship behavior), that are between the level elicited by virgin WT females & mated WT females. Virgin females expressing sra[GS3080] under the control of Scer\GAL4$^{elav\text{-}C155}$ have a high extrusion index (EI, the percentage of a time a female shows extrusion behavior when being courted by a male) which is nearly comparable to that of WT mated females. Homozygous females lay fewer eggs than WT & most of the eggs are arrested at metaphase I of meiosis. Spontaneous ovulation occurs in 3% of mutant virgin females (virtually no ovulation occurs in WT virgin females). The level of ovulation of mutant mated females is not as high as that of WT mated females. The mutant females show reduced receptivity after mating (as occurs in WT females), but their remating rate 24 hrs after mating is significantly higher than that of WT females. Receptivity of mutant females is restored earlier than for WT females; the remating rate is significantly higher for mutant females compared to WT females 10 days after mating.	
suppressor of forked	*su(f)*	Males do not appear sufficiently energetic to mate.	
Cyp4d21	*sxe1*	Decreased male courtship & mating success	Fujii *et al.* 2008 Genetics 180:179-90

Table A1. Continued

Gene	Symbol	Phenotype	Reference
Sex-lethal	*Sxl*	$Sxl^{M1,f3}/Sxl^{f7,M1}$ males behave like normal males. $Sxl^{M1,f3}/Sxl^{f7,M1}$ females elicit less courtship than normal females & produce large quantities of the inhibitory pheromones that normal males synthesize. Mutant females also produce very little or none of the female-predominant aphrodisiac pheromone.	
takeout	*to*	When cells that normally express takeout are feminized by expression of the Transformer-F protein, male courtship behavior is dramatically reduced, suggesting that male identity in these cells is necessary for behavior. A loss-of-function mutation in the takeout gene reduces male courtship & synergizes w/ fruitless mutations, suggesting that takeout plays a redundant role w/ other fru-dependent factors involved in male mating behavior.	Dauwalder *et al.* 2002 Genes & Development 22:2879-92
touch insensitive larva B	*tilB*	Mutants show complete absence of sound-evoked courtship behavior. The sound evoked response (measured via the antennal nerve) is eliminated. In mutant alleles the spermatid axonemes are defective. Mutant males demonstrate vigorous courtship including courtship songs. Relative amplitude of the sine song is higher than normal in mutant male songs.	
timeless	*tim*	If either male or females are mutant, this abolition of mating rhythm is seen, even if they are mated to WT flies. Single tim^{01} mutant females mated once to single tim^{01} mutant males lay significantly less eggs & produce significantly less progeny than WT flies. The percentage of unfertilised eggs from this mating is significantly greater than for WT. These effects on fertility are significantly reduced if pairs of per^{01} mutant flies are allowed to mate over a period of 4 days. Single WT females mated once to single tim^{01} mutant males lay significantly less eggs & produce significantly less progeny than those mated to WT males. The numbers of sperm released to the seminal vesicles by tim^{01} males over the 2 days following eclosion is significantly less than that seen in WT males. Single tim^{01}/tim^{03} females mated once to single tim^{01}/tim^{03} males produce significantly less progeny than do WT flies, even though the number of eggs laid & the percentage of fertilised eggs is not significantly different to WT. tim^{01}/tim^{03} males have significantly less sperm in their seminal vesicles 42 hrs after eclosion than WT males. The time spent copulating is significantly increased in tim^{01} mutant males mated to WT females, compared to WT males. This effect is seen in both 2 day old & 4 day old males. This increased time in copulation does not appear to be due to difficulty of males disengaging from females. The time spent copulating by tim^{01}/tim^{03} males mated w/ WT females is significantly extended compared to WT. Long term memory of courtship conditioning (reduction in time spent in courtship behavior 5 days after a 7 hour conditioning) is normal in tim^{01} homozygous males.	
temperature-induced paralytic E	*tipE*	The courtship song parameters interpulse interval & cycles per pulse are normal in homozygous males at 25°C, but the interpulse frequency & amplitude of sound are significantly lower than in WT males at 25°C.	
technical knockout	*tko*	Mutant males are rejected by WT females. They are moderately successful in courting mutant females, but show a substantially prolonged courtship & reduced mating time compared to WT. WT males are equally successful at courting either mutant or WT females. Mutant males show only a very slight response to courtship song at 100dB, whereas WT males show a clear response at 70-90dB, suggesting a hearing defect in mutant males.	Toivonen *et al.* 2001 Genetics 159:241-54

Table A1. Continued

Gene	Symbol	Phenotype	Reference
transformer2	*tra2*	The genital discs tra2$^{dsRNA.Scer\UAS}$; Scer\GAL4$^{ptc\text{-}559.1}$ females raised at 29^{o}C resemble male genital discs, & are often indistinguishable from those of their male siblings. However, this phenotype is not completely penetrant: some growth of the female primordia is seen in a minority of cases. When tra2$^{dsRNA.Scer\UAS}$ is driven by Scer\GAL4$^{fru.P1}$ in females, the initial stages of courtship behavior - orientation & tapping - are seen when paired w/ a WT virgin female, but wing & proboscis extension & attempted copulation are not seen. When paired w/ a WT male these masculinized females are always courted, but show male-like rejection behaviors, including wing flicking & kicking, & never show the female rejection response of ovipositor extrusion. When a single WT male is placed w/ multiple masculinized tra2$^{dsRNA.Scer\UAS}$, Scer\GAL4$^{fru.P1}$ females, male singing is sufficient to elicit wing extension & vibration as well as occasional proboscis extension in a mutant female not being courted.	
Vesicular monoamine transporter	*Vmat*	Homozygous females are essentially infertile & do not appear to lay any eggs as virgins or after mating. Only 21% of homozygous males produce adult progeny when mated w/ WT females. The ovaries of 3 day old, mated homozygous females contain 3.5 fold more mature oocytes than control ovaries. The mutant ovaries are similar in length & only slightly larger than control ovaries despite the dramatic increase in oocyte content. The ovaries of 3 day old, virgin homozygous females contain fewer mature oocytes than control ovaries from WT virgin females (which retain mature oocytes in their ovaries) & the mutant virgins show a modest decrease in ovary size compared to controls.	
Voila	*V*	Heterospecific effect	Grosjean *et al.* 2001 Genetical Res 77:239-50
yellow	*y*	Males have a reduced wing extension index (the percentage of each courtship ritual during which a male's wing is extended & vibrating) compared to WT. y^{1} males have significantly reduced mating success compared to control males.	

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EDUCATION

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Fall 2006-Summer 2008	Graduate Teaching Assistant, Critical Writing in Biology
Fall 2004-Spring 2005	Graduate Teaching Assistant, Introductory Biology I Lab
2001-2005	Substitute Teacher, Deer Park Independent School District Specializing in Pre-AP and AP science courses

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2009-2010	Biology Graduate Student Association President
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2010	Outstanding Teaching Award, Dept of Biology, Texas A&M University
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PUBLICATIONS

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