Socially-responsive gene expression in male *Drosophila melanogaster* is influenced by the sex of the interacting partner

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Abstract

Behavior is influenced by an organism’s genes and environment, including its interactions with same or opposite sex individuals. *Drosophila melanogaster* perform innate, yet socially modifiable, courtship behaviors that are sex specific and require rapid integration and response to multiple sensory cues. Furthermore, males must recognize and distinguish other males from female courtship objects. It is likely that perception, integration, and response to sex-specific cues is partially mediated by changes in gene expression. Reasoning that social interactions with members of either sex would impact gene expression, we compared expression profiles in heads of males that courted females, males that interacted with other males, or males that did not interact with another fly. Expression of 281 loci changes when males interact with females, while 505 changes occur in response to male-male interactions. Of these genes, 265 are responsive to encounters with either sex and 240 respond specifically to male-male interactions. Interestingly, 16 genes change expression only when a male courts a female, suggesting that these changes are a specific response to male-female courtship interactions. We supported our hypothesis that socially responsive genes can function in behavior by showing that *egghead (egh)* expression, which increases during social interactions, is required for robust male-to-female courtship. We predict that analyzing additional socially-responsive genes will give us insight into genes and neural signaling pathways that influence reproductive and other behavioral interactions.
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 behavioral interactions to gain insight into corresponding physiological changes. 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 social interactions with females (Carney 2007; Ellis and Carney 2009). However, we do not know if these are specific responses to females or more general responses to interacting with a second individual. 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 (reviewed in 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 (reviewed in Cline 2005; Shirangi and McKeown 2007) and sexually dimorphic development, 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 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 or other social interactions, 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, interacting with a second individual 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 expression analysis 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.

Since our earlier studies did not address the possibility that some of the loci that respond to male-female interactions might be generally “socially-responsive” rather than specifically “courtship-responsive” genes, we examine this possibility in the current study by examining gene expression changes occurring in the male head (rather than whole body) during interactions with either females or males. We also expanded on our earlier studies by showing that socially-responsive loci can function in behavior. Our data indicate that social interactions cause expression changes in loci expressed in neuronal tissue as well as in non-neuronal adipose tissue that may modulate neural signaling and 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 two fine tungsten wires on the external genitalia of the female to prevent mating. Females recovered for 1 day in a new vial. All flies were kept on a 12-hr light/dark cycle at 25°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 5 included equally dividing males into three groups: (1) Courting male, (2) Male-male and (3) Control. For the courting male treatment, one cauterized female was aspirated into a male’s vial. For the male-male group, a second male was aspirated into the vial of a single male. Control males were treated the same except that a second fly was not transferred during the aspiration process. Courtship or male-male exposure lasted for 20 min. In courting male treatments the presence of courtship was assessed at 1 min intervals. Only males that courted a female for at least 70% of the observation time were collected for analysis. During this time brief male-male interactions (lasting only a few seconds) were observed. We did not detect locomotor differences among males in the 3 treatment groups (two-tailed t-test, p>0.05). After 20 min, males were removed from the vials, quick frozen in liquid Nitrogen, and stored at -80°C for future RNA extraction.

We separated heads from the rest of the bodies by vortexing quick-frozen flies. For each treatment, 20 male heads were randomly assigned to 1 of 10 groups, giving us 10 RNA preparations for each of the courting male, male-male or control treatments. 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 RNA preparations each from courting, male-male and control heads (15 individual samples) to Affymetrix Drosophila 2.0 Genome Arrays following standard Affymetrix (Santa Clara, CA, USA) protocols. Therefore, animals for all 3 treatment groups were collected and analyzed at the same time, and all 15 microarray hybridizations were carried out concurrently.

We extracted expression values from the microarrays using 5 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). For paired data analysis comparing courting male and control treatments, we conducted Bayesian $t$-test (CyberT, Baldi and Long 2001) and false discovery rate analyses ($q<0.05$, Storey and Tibshirani 2003), requiring that $p<0.001$ in at least 3 of 5 algorithms. For a combined analysis of the 3 data sets we used the SAS Mixed procedure (SAS Institute Inc., Cary, NC, USA) and identified significantly up- and down-regulated socially-responsive genes ($p<0.05$ in at least 3 of 5 algorithms).

Courtship-responsive genes are those for which expression in courting male heads differs from that in control and male-male heads (control = male-male expression). Male-male-responsive genes are those that differ only in male-male interactions (control = courting male expression). Other genes that respond to interactions with both sexes were placed in the general category of socially-responsive genes.

$q$ PCR
We validated the microarray results by qPCR analysis on the 5 control and 5 courting male RNA preparations 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).

Since few of the socially-responsive loci identified from the paired analysis (courting male compared to control) had known or predicted functions in behavior, primers were designed for a randomly chosen set of 6 up-regulated (CG9377, CG10621, erguson, HLHmβ, Lsp2, sug) and 3 down-regulated (CG31181, Rim, Sh) candidate genes. We chose a range of genes with adult expression predicted to be enriched in the brain (CG9377, Rim, Sh), fat body (Lsp2, sug), or both tissues (CG10621, CG31181, HLHmβ, erguson (erguson expression is very low in fat)) (Chintapalli et al. 2007). Genes with low predicted transcript levels in the head were not tested (Chintapalli et al. 2007). Of these selected genes, only erguson and Sh had previously described reproductive behavioral roles in females and males, respectively. 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.

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.

A regression analysis of microarray mean expression fold changes compared
to independent qPCR fold changes indicated a significant positive correlation
between results obtained by both methods \( (r = 0.68, N = 9, p= 0.006) \).

**In situ hybridization**

We performed *in situ* hybridization for a subset of socially-responsive genes
using cDNA clones for CG9377 (GH08193), CG10621 (RE64786), cwo (LD15411),
egend (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 (brains, heads or abdominal carcasses) as
previously described (Arbeitman *et al.* 2004).

To confirm that fit expression increased in courting males compared to control
males, we generated antisense and sense probes directed against fit using the
RH40291 clone. Control and courting male heads were cryosectioned and incubated
with fit probes as described above. We only detected signal using antisense probes.
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 (\textit{egh}^{EP804}, \textit{egh}^{FY03917}). Both insertions are located within the first \textit{egh} exon and reduce \textit{egh} expression to barely detectable levels (Fig. S1). For both X-linked P-element insertions, we crossed P-element females to isogenic \textit{CS} males, and we crossed P-element males to isogenic \textit{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 \textit{CS} virgin females (20 or fewer) were aged collectively for 3 to 5 days. All courtship tests with \textit{egh} mutants were performed in dim red light conditions because mutations in \textit{egh} affect photoreceptor pathfinding (Fan \textit{et al.} 2005) and therefore likely impact eye function. In red light conditions, fly courtship relies more heavily upon sensory systems other than the eye.

We analyzed courtship behavior under red light at 22°C. A male was aspirated into a mating chamber (diameter = 1 cm) and a virgin \textit{CS} female was introduced 2 min later. The pair was video recorded for 10 min. The courtship index (CI; time performing courtship divided by the total observation time) was calculated. CI values were arcsine transformed and two-tailed \textit{t}-test comparisons between mutants and controls were calculated to determine significance (p<0.05).

To reduce \textit{egh} specifically in the adult nervous system we utilized two \textit{egh}-RNA interference (RNAi) alleles, \textit{egh}^{v45160} and \textit{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 (Fig. S1).

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 (Fig. S2). 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-Gal80ts (reviewed in McGuire et al. 2004). Crosses were maintained at the permissive temperature. Control males had UAS-Dicer-2 and UAS-tubulin-Gal80ts 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) to egh$^{EY03917}$ and compared CIs of egh$^{EY03917}$; eghP2 males to egh$^{EY03917}$ males. 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. egh$^{EY03917}$ males with either component of the Gal4/UAS system served as controls. Both rescue experiments were carried out at 22°C under red light.
Results

Changes in male gene expression during social interactions with females or males

Within 5 min of male-to-female social interactions, whole-animal transcript profiles are altered in courting males, and there is a differential response to conspecific compared to heterospecific females (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 loci with altered expression due to male-female interactions (See Materials and Methods). Sixteen transcripts were up regulated (Table 1) and 19 were down regulated (Table 2) 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). The small number of loci with altered expression is consistent with results from other behavioral studies (e.g., Lawniczak and Begun 2004; Mack et al. 2006; Wada et al. 2006) and is partially a consequence of our extremely conservative criteria for identifying responsive genes (See Materials and Methods).
We performed a second analysis of our data that included a third comparison to gene expression changes occurring as a consequence of male-male interactions. This strategy allowed us to distinguish loci whose expression changes due to interactions specifically with females from those that change due to interaction with another individual of either sex (see Materials and Methods). Five hundred-five genes responded to male-male interactions, while 281 genes responded to male-female interactions. Most expression changes that occur due to male-female interactions also occur as a consequence of male-male interactions (Table S1). This list of 265 genes includes 24 genes present in the original comparison between courting male and control heads. We also identified 240 genes whose expression changes specifically in response to paired male interactions (Table S2).

Sixteen genes were responsive to male-female interactions but not male-male interactions and, therefore, appear to be true courtship-responsive loci (Table 3). Five of these 16 loci (Drop, sugarbabe (sug), hairy, olf186-M, HLHmβ) also were present on the list from the paired comparison of courting males to control males. Six genes with strong statistical support in the initial comparison of courting males and control males (egh, Lsp2, clockwork orange (cwo), cacophony (cac), CG2217, CG4629) were not present in the new list of genes that respond to encounters with both sexes. However, cwo is on the list specific to male-male interactions (Table S2), suggesting that it may be socially responsive. It is possible that expression changes in the remaining 5 genes from the paired comparison are also a specific response to courtship rather than a general response to social interactions. In support of this argument, cac functions in production of male courtship song. We
refer to the broad group of genes identified here as “socially-responsive genes” and refer to specific subcategories (e.g., courtship-responsive, male-male-responsive) as appropriate.

qPCR validation of microarray results

To verify our microarray results, we used qPCR to analyze transcript levels from a subset of socially-responsive genes from Tables 1 and 2. We compared expression in control and courting male head RNA preparations not used for microarray hybridization. The 6 up-regulated and 3 down-regulated socially-responsive genes tested showed the expected trends in expression (Table 4; See also Materials and Methods) with fold changes comparable to those from the microarrays. Four of the genes, including the courtship-responsive loci sug and HLHmβ, showed statistically significant changes in courting males compared to controls. Since all 9 genes showed the expected trend by qPCR, this is strong support for the validity of the microarray data. Increasing the sample sizes would likely increase the statistical support.

Socially-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 many socially-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 socially-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 S3).

Two socially-responsive genes, Lsp2 and female-specific independent of transformer (fit) are expressed in fat surrounding the brain in both sexes (Benes et al. 1990; Fujii and Amrein 2002). fit was named for 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).

In an earlier whole-male microarray analysis, we detected a statistically significant increase in fit transcripts in courting males; this increase was validated by qPCR (Carney 2007). fit also increases in 20 min courting male heads (Table 1) and is responsive to same-sex interactions as well (Table S1). Since we had not examined the specific tissue in which this increased expression occurs, we examined fit’s response to 20 min courtship in sectioned male heads. Although expression is low in virgin males, fit levels increased in response to male-female interactions (Fig. 1). This increase was detected in the fat body, an adipose tissue previously implicated in modulation of courtship behavior (reviewed in Dauwalder 2008). In situ hybridization confirmed that other socially-responsive genes are expressed in the male fat body (CG10621, sug), the male brain (CG9377, egh), or both tissues (cwo) (Figs. 2 & 3).

egghead is required in the adult male brain for robust courtship

We hypothesized that genes with altered expression patterns due to male-female interactions 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 increased 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.

Therefore, we tested P-element insertions or Vienna Drosophila RNAi strains targeting some of the male-female-responsive genes from Tables 1 and 2 for effects on male courtship activity (measured as the courtship index, CI). For several of the alleles, we observed weak phenotypic effects on behavior. However, mutations in egh had strong effects on male-female courtship, so we focused our current downstream analysis on this locus. Males with either of 2 independent insertions in egh (egh$^{EP804}$ and egh$^{EY03917}$) performed all standard courtship behaviors but had significantly reduced CI values compared to genetically similar controls (Fig. 4, two-tailed t-test, p<0.001). We did not observe male-male courtship or aggressive interactions in groups of aged mutant males that were placed together in vials and observed over a two-week period. Therefore, reduced egh expression led to an overall reduction in time spent courting a female but did not appear to affect male-male interactions. Reintroduction of a genomic copy of egh in the egh$^{EY03917}$ background restored male-to-female courtship activity to wild-type levels (Fig. 5, p<0.001), verifying that the courtship phenotype is due to disruption of the egh locus.

We selectively reduced egh in the adult nervous system with UAS-egh-RNAi under the control of UAS-tubulin-Gal80ts$^{6}$ and neural-expressed elav$^{155}$-Gal4. This adult-specific decrease in egh resulted in significantly reduced CI values for
experimental males at the restrictive temperature (29°C) compared to all controls (Fig. 6).

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). We asked whether this same circuit functioned in male reproductive behavior. Expressing *egh* (via *UAS-eghHA*) under control of *ap<sup>md544</sup>*-*Gal4* in *egh*<sup>EY03917</sup> mutant males was sufficient to restore male courtship behavior (Fig. 7, *p*<0.001), indicating that Ap neurons modulate reproductive behaviors in both sexes. Though Soller *et al.* (2006) attributed modulation of the Sex-peptide response to developmental expression, *ap<sup>md544</sup>*-*Gal4* expresses in the male and female adult nervous system (Fig. S2). Therefore, we expressed *egh*-RNAi via *ap<sup>md544</sup>*-*Gal4* to specifically reduce *egh* expression in adult males (Fig. 8). Targeted *egh* reduction significantly decreased courtship activity (*p*<0.001), confirming that *egh* is needed in Ap neurons during adulthood for proper courtship behavior.

**Discussion**

**Social interactions alter male gene expression**

*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 male-female social interactions cause rapid (within 5 min) changes in whole-male transcript abundance (Carney 2007). In this study we focused on male head tissue and found that 521 genes are socially responsive in a 20 min interaction period.
Expression of 281 genes changes during male-female interactions, while 505 genes are affected by male-male interactions. At least 16 of these loci are specifically courtship responsive (Table 3). Similarly to genes identified in array studies on songbirds and honey 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), the 16 Drosophila courtship-responsive genes include several loci that regulate gene expression and neural development and signaling, but their specific relationship to behavior is not clear. These loci may control gene cascades important for subsequent courtships, such as those that fine-tune neural connections due to courtship and mating experience. An additional set of 5 genes identified only in a paired comparison of courting and control males (Lsp2, egh, cac, CG2217, and CG4269) may also be courtship-responsive since their transcript levels were not affected by male-male interactions. If not specifically courtship responsive, they are likely to be generally socially responsive or to have behavioral functions. In support of this hypothesis, we showed that egh expression is important for robust male-female courtship.

Interestingly, a much larger group of genes is responsive to interactions with both sexes (265 genes, Table S1) or is male-male responsive (240 genes, Table S2). Therefore, social interactions have an impact on gene expression patterns that depends on the sex of the interacting individuals. This result is not surprising since social experience affects a variety of behaviors and morphological phenotypes in flies and other animals (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; Technau 2007). Some of the expression changes identified in our study may underlie observed effects of social interactions on circadian behavior and pheromone profiles (Levine et al. 2002; Kent et al. 2008; Krupp et al. 2008).

The large number of male-male responsive genes was surprising. However, male-male interactions such as those involved in aggressive encounters may have greater effects on gene expression than male-female interactions. Males of many Drosophila species, including Drosophila melanogaster, compete for mates and territories, and aggressive behavior is correlated with mating success (Dow and von Schilcher 1974); both factors correlated with genotype (Cabral et al. 2008). Social experience with other males reduces aggressive behavior during competition for territories, and experienced males are more likely to regain territories (Hoffman 1990). Although our male-male assays were performed under conditions under which there is predicted to be little male-male competition (e.g., no food source or female), it is likely that sensory processing and gene expression was affected by the brief encounters between individuals. Observed changes in gene expression due to male-male interactions may contribute to the phenotypic plasticity in behaviors important for obtaining territories, food sources, and mates.

Further investigation is required to understand fully the importance of the large number of changes that occur due to general social interactions or specifically in response to male-male interactions.

Socially-responsive genes and the sex-determination hierarchy
We predicted that some socially-responsive loci would function as downstream targets of the somatic sex-determination pathway that regulates male courtship behavior. Three transcription factors, Fruitless (Fru), Doublesex (Dsx), and Dissatisfaction (Dsf), are important regulatory components of this pathway. One courtship-responsive gene, \textit{CG13116}, is negatively regulated by the female-specific Doublesex (Dsx) protein, and one up-regulated male-male-responsive gene, \textit{CG16713}, is downstream of \textit{transformer (tra)} (Goldman and Arbeitman 2007). Four up-regulated socially-responsive genes are regulated by the sex-determination pathway. \textit{fit} is regulated by \textit{tra}; \textit{CG9377} is downstream of \textit{fru}; and \textit{CG9837} and \textit{CG8539} are regulated by \textit{dsx} (Goldman and Arbeitman 2007). The surprisingly small number of socially-responsive genes that are known sex-determination hierarchy targets may indicate that our lists include many target genes that could not be detected by the strategies used previously to identify output genes of the hierarchy. For instance, genes from our study may function downstream of \textit{dsf}, which is expressed in both males and females; transcriptional targets of \textit{dsf} are not known. Another possibility is that the hierarchy does not regulate expression of many socially-responsive genes, indicating an alternative regulation.

Gene expression in the male brain

Since brain gene expression has a clear function in behavior, we expected some socially-responsive genes to be expressed in the brain. \textit{In situ} hybridization showed that \textit{CG9377} and \textit{egh} were expressed in the male brain but were not detected in adipose tissue (Figs. 2 & 3). Two down-regulated, socially-responsive
genes function in courtship behavior and are expressed in the brain. *cac* encodes a calcium voltage-gated channel needed for courtship song production (reviewed in Billeter *et al.* 2002; Greenspan and Ferveur 2000); *Shaker* (*Sh*) encodes a potassium channel that functions in olfactory memory and learning (reviewed in Greenspan and Ferveur 2000). Other socially-responsive genes (*e.g.*, *Drop*, *egh*, *hairy*, 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 may modulate adult neural signaling and behavior.

Changes in brain gene expression patterns due to social 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 social 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 growing 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 (Fig. 1) (Benes et al. 1990; Fujii and Amrein 2002), and in situ hybridization confirmed fat body expression of 3 additional socially-responsive genes (CG10621, cwo, and sug) (Fig. 3). cwo is also expressed in the male brain, but we did not detect CG10621 or sug transcripts in the male brain. Many socially-responsive genes are enriched in head tissue, including fat body but not including the brain (Figs. 2 & 3; Table S3). This suggests that the circuitry responding to and governing social interactions such as courtship likely is modulated by both neuronal and non-neuronal signals. The response to social interactions involves complex and specific changes that may mediate various downstream effects including neural plasticity.

**egghead and courtship behavior**

To determine if mutations in candidate genes affected courtship behavior, we measured CI values in various mutants. Our analysis showed that a specific locus, egghead, is needed for robust male courtship behavior (Fig. 4). egghead encodes a β1,4-mannosyltransferase which regulates glycosphingolipid biosynthesis (Wandall et al. 2003), affects Drosophila neural development and behavior, and is required in Ap neurons for female Sex-peptide response post mating (Soller et al. 2006).

Since ap-md544-Gal4 (a Gal4 insertion in ap) is expressed in the adult nervous system of both sexes (Fig. S2), we examined whether this neural circuit also functioned in males to regulate courtship behavior. In eggheadEY03917 mutant males, egghead expression in Ap neurons rescued the courtship defect (Fig. 7). Decreased adult egghead expression (via RNAi) in ap-expressing neurons also resulted in decreased courtship
Although Fru neuron expression of the EcR transcription factor is important for courtship behavior (Dalton et al. 2009), decreasing EcR in adult Ap neurons did not affect courtship (Figs. S3 & S4). Therefore, egh appears to have a specific behavioral function in Ap-expressing neurons. Ap is a transcription factor that regulates developmental as well as post-developmental neural gene expression (Benveniste et al. 1998). ap mutant males also have decreased levels of male-to-female courtship (Ringo et al. 1992). Given the similarity between the ap and egh mutant phenotypes and the requirement for egh expression in ap neurons for male courtship, the hypothesis that ap regulates egh expression should be tested in future experiments.

Differences in sex-specific 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). 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 both male and female Ap neurons 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 eghRNAi in fru neurons did not cause the behavioral defects observed in egh mutant or apmd544-Gal4/eghRNAi males (data not shown).
Therefore, *egh* neurons may interact indirectly with *fru* neurons to modulate 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 socially-responsive loci, we can clarify the relationship between genetics and the intracellular processes governing behavior and physiology. Additional studies are needed to understand the relationship of courtship-responsive and other socially responsive loci to the somatic sex-determination hierarchy or other pathways that regulate Drosophila reproductive behaviors.

**Authors’ contributions**

GEC conceived and designed experiments and helped write the manuscript. LLE designed and executed experiments, analyzed the data, and helped write the manuscript.

**Acknowledgements**

We thank Ms. Donna Wall and Dr. Kuey-Chu Chen at the University of Kentucky MicroArray Core Facility for microarray processing, Mr. Bruce Ellis for creating the electrical probes used for cauterization, Dr. Matthias Soller for providing *egh*P2 and *UAS-egh*HA, and the VDRC for providing the *egh-RNAi* and *UAS-Dicer2* alleles. We thank two anonymous reviewers for comments on the manuscript. Texas
A&M University provided funding to G.E.C. The microarray data from this study are available through the GEO database via accession number GSE24167.

References


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Fig. 1: Courting males show increased *fit* expression in the fat body. A DIG-labeled *fit* RNA antisense probe was made from the RH40291 cDNA clone. *In situ* hybridization was performed on cryosectioned male heads and confirmed that *fit* transcript levels are up regulated in the adipose tissue (arrows) of courting males (A) compared to control males (B). A qualitative assessment of signal intensity in both treatment groups is presented in panel C.

Fig. 2: Socially-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. Dark pink staining in panels A, C and E indicates expression due to hybridization of antisense probes.

Fig. 3: Socially-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. Expression is indicated by light (*cwo*) or dark purple (*CG10621, sug*).
Figure 4. *egh* is required for robust male courtship behavior. Under red light, males with either X-linked *egh* insertion (*egh*<sup>EP804</sup> or *egh*<sup>Y03917</sup>) show significant (***p<0.001) decreases in CI values compared to control males in a similar genetic background (CS(*egh*<sup>EP804</sup>) or CS(*egh*<sup>Y03917</sup>)) under similar conditions. Error bars reflect the SEM. N=10 males for each genotype.

Figure 5. *egh* expression rescues male courtship behavior. Restoring *egh* expression in *egh*-expressing cells (*egh*P2) in the *egh*<sup>Y03917</sup> mutant background significantly (***p<0.001) rescued the courtship defect in *egh*<sup>Y03917</sup> mutant males. N=10 males for both genotypes.

Figure 6. Male courtship requires *egh* expression in the adult nervous system. Expressing UAS-*egh*-RNAi alleles, *eghv45160* or *eghv45161*, in the adult nervous system using elav<sup>c155</sup>-Gal4, UAS-Dicer-2, and UAS-tubulin-Gal80<sup>ts</sup>, at the restrictive temperature (29°C, black bars) significantly (**p<0.01;*p<0.05) reduced male courtship activity compared to 29°C controls lacking elav<sup>c155</sup>-Gal4 or UAS-*egh*-RNAi compared to males at the permissive temperature (20°C, gray bars).

Figure 7. *egh* expression in *ap*-expressing neurons restores male courtship behavior. Narrowing *egh* expression to *ap* neurons by expressing UAS-*egh*HA under the control of *ap*<sup>md544</sup>-Gal4 in the *egh*<sup>Y03917</sup> background significantly (***p<0.001) restored male courtship activity compared to control *egh*<sup>Y03917</sup> males.
lacking either component of the Gal4/UAS system. Ten males of each genotype were tested.

**Figure 8. Adult expression of egh in ap-expressing neurons is necessary for robust courtship behavior.** Expressing UAS-egh-RNAi alleles, egh\(^{v45160}\) or egh\(^{v45161}\), in Ap neurons during adulthood using ap\(^{md544}\)-Gal4, UAS-Dicer-2, and UAS-tubulin-Gal80\(^{ts}\) at 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).
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<thead>
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<th>-</th>
<th>+</th>
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<td>10</td>
<td>3</td>
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<td>(B) Control males</td>
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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.
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Average fold changes, molecular functions and biological processes are shown for 19 genes that are significantly (p<0.001) down regulated in male heads after 20 min of courtship.
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<td>CG32491</td>
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<td>-1.09 -1.16</td>
<td>Transcription factor activity</td>
<td>Induction of apoptosis</td>
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Eleven up-regulated and 5 down-regulated genes are courtship-responsive when comparing all three treatment groups (control, courting and male-exposed) by mixed ANOVA and Tukey's post-hoc analyses (p<0.05).
<table>
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<th>Gene identifier</th>
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<th>Avg. relative expression level in control male heads±SEM</th>
<th>Avg. relative expression level in courting male heads±SEM</th>
<th>Relative qPCR fold change ±SEM</th>
<th>Relative array fold change ±SEM</th>
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Average relative expression of nine genes was assessed by qPCR. * Indicates a significant (p<0.05) difference in the average relative expression level in control male heads compared to courting male heads as determined by qPCR. SEM=Standard error of the mean.