A genome-wide analysis of courting and mating responses in *Drosophila melanogaster* females

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Abstract: In *Drosophila melanogaster*, seminal fluid proteins influence several components of female physiology and behavior, including re-mating rates, ovulation and oviposition, and sperm use. It is well-known that female flies are not simply passive vessels and that female-mediated interactions with male products are important to female (and thus male) reproductive success. While the population genetics, molecular evolution and physiological effects of seminal fluid proteins have been examined, the genetics and evolution of the female side of these post-mating interactions is unexplored in spite of work showing that female genotype and female-by-male genotype interactions are important determinants of sperm competition outcomes. Here we use microarrays to identify candidate genes involved in the female side of post-mating sexual interactions. We report the results of a whole-genome oligonucleotide chip experiment that reveals 23 genes differentially expressed between virgin females exposed and unexposed to courting males, and 38 genes differentially expressed between virgin and recently mated females. Immune related genes are overrepresented among the mating-influenced candidates. We use quantitative reverse-transcriptase PCR to independently assess gene expression changes for roughly half of the mating-affected candidate genes.

Key words: reproduction, gene expression, Drosophila immune related genes, serine proteases, accessory gland proteins.

Résumé : Chez *Drosophila melanogaster*, les protéines du liquide séminal influencent plusieurs composantes de la physiologie et du comportement de la femelle, soit le taux de ré-accouplement, l'ovulation, l'oviposition et l'utilisation de sperme. Il est bien connu que les mouches femelles ne sont pas simplement des réceptacles passifs, mais que les interactions dépendantes de la femelle avec les produits du mâle sont importantes pour le succès reproducteur de l'espèce. Alors que la génétique des populations, l'évolution moléculaire, et les effets physiologiques des protéines du liquide séminal ont été examinés, les aspects génétiques et évolutifs des interactions post-accouplement d'un point de vue de la femelle sont inexplorés malgré les travaux qui démontrent que le génotype de la femelle et que les interactions génotypiques femelle-par-mâle sont des déterminants importants pour le résultat de la compétition au sein du sperme. Nous avons utilisé des micro-puces pour identifier des gènes candidats impliqués dans les interactions post-accouplement du point de vue de la femelle. Nous rapportons les résultats obtenus sur des puces d'oligonucléotides couvrant le génome entier qui révèlent 23 gènes exprimés de façon différentielle entre des femelles vierges exposées et non exposées à des mâles courtisans et 38 gènes exprimés de façon différentielle entre des femelles vierges et des femelles récemment accouplées. Des gènes reliés à l'immunité sont sur-exprimés parmi les gènes candidats influencés par l'accouplement. Nous avons utilisé la réaction de polymérase en chaîne quantitative couplée à la transcriptase inverse afin d'évaluer indépendamment les changements dans l'expression génétique pour environ la moitié des gènes candidats affectés par l'accouplement.

Mots clés : reproduction, expression génique, gènes reliés à l'immunité chez la drosophile, protéases à sérine, protéines des glandes accessoires.

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Introduction

Mating causes dramatic changes in female physiology and behavior that directly affect reproductive success. *Drosophila melanogaster* females mate multiply and store sperm in paired spermathecae and the seminal receptacle (Gromko and Markow 1993; Harshman and Clark 1998; Imhof et al. 1998). Copulation in this species typically lasts about 20 min during which 4000–6000 sperm are transferred (Kaplan et al. 1962). However, <1 000 sperm are stored and only 300–800 are actually used for fertilization (Kaplan et al. 1962; Fowler 1973). *Drosophila melanogaster* males also

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transfer approximately 100 different seminal fluid proteins produced by the accessory glands, ejaculatory duct and ejaculatory bulb during copulation (Wolfner et al. 1997; Swanson et al. 2001). Accessory gland proteins, known as Acps, have important fitness consequences for both sexes. Females mated to mutant males that transfer sperm but no Acps produce no offspring, suggesting that Acps are essential for fertility (Xue and Noll 2000).

Though Acps are essential for fertility, they reduce female lifespan (Chapman et al. 1995). A cost to females has also been suggested by experimental evolution studies showing that males can evolve greater or lesser toxicity depending on the mating system and that females can evolve resistance to the toxic effects of males (Rice 1996; Holland and Rice 1999). This type of variation suggests the presence of continuous adaptation and counter-adaptation between the sexes.

The effects of several Acps on female physiology and behavior are known. Sex Peptide (Acp70A) stimulates oogenesis and represses female receptivity to re-mating (Chen and Balmer 1989; Schmidt et al. 1993). In 2 recent studies using targeted mutagenesis and RNAi, Sex Peptide was shown to be the major protein responsible for both the short and long-term maintenance of these phenotypes in females (Liu and Kubli 2003; Chapman et al. 2003). Sex Peptide binds to sperm tails and may be released when a sperm fertilizes an egg, thus, maintaining increased oogenesis and refractoriness (Liu and Kubli 2003). This protein may also stimulate increased juvenile hormone levels and increased yolk protein production and uptake into oocytes (Moshitzky et al. 1996; Soller et al. 1997). Ovulin, also known as Acp26Aa, stimulates the release of oocytes (Herndon and Wolfner 1995; Prout and Clark 1996). A null allele of Ovulin results in fewer eggs oviposited during the first 24 h after mating (Herndon and Wolfner 1995). However, a greater fraction of the eggs deposited during this time are fertilized (Chapman et al. 2001). Ovulin population variation was correlated with sperm displacement in *D. melanogaster* (Clark et al. 1995). Interestingly, Ovulin, though male-derived, shows amino acid similarity to an egg-laying-hormone produced by *Aplysia californica* females (Wolfner 1997). Another Acp, Acp36DE, is required for proper sperm storage. Females mated to mutant males lacking Acp36DE store only 15% as many sperm as females mated to wild-type males (Neubaum and Wolfner 1999).

In some cases, the physiological targets of Acps within females are known. Sex Peptide crosses the posterior vaginal wall and enters the hemolymph during the first 10 min of copulation (Lung and Wolfner 1999). It then binds to nervous tissue in the female, suggesting that its effects could be a result of interaction with the female nervous system (Ottiger et al. 2000; Ding et al. 2003). Approximately half of the amount of transferred Ovulin protein remains in the reproductive tract where it is cleaved into several forms. The remainder enters the female hemolymph and localizes to the base of the ovary where it likely causes nervous or hormonal stimulation of ovulation (Monsma et al. 1990; Park and Wolfner 1995; Lung and Wolfner 1999; Heifetz et al. 2000). Acp36DE associates with sperm and localizes to the openings of the sperm storage organs as well as within these organs (Bertram et al. 1996; Neubaum and Wolfner 1999).

Laboratory experiments on extracted chromosome lines derived from wild-caught flies show that there is abundant variation expressed in females that affects the outcome of sperm competition (Clark and Begun 1998). Moreover, the outcome of sperm competition can depend on unpredictable, strong genetic interactions between female and male genotypes (Clark et al. 1999). Thus, data from functional experiments and from surveys of variation among natural genotypes support the notion that seminal fluid proteins interact with specific pathways in females to affect physiology, behavior, and patterns of sperm use. However, virtually nothing is known about the genetics or population genetics of the most fundamental female components of postmating biology such as sperm storage, ovulation, and fertilization (Bloch Qazi et al. 2003; Civetta 2003). To begin to address this gap, we report here our genome-wide comparative analysis of transcription in virgin females, courted virgin females, and mated females (2 h post-copulation).

Methods

Microarray experiment treatments

The *D. melanogaster* stock used for the experiments was a healthy, highly inbred line (WI-96, courtesy of Dr. Sergey Nuzhdin) derived from a female caught in the Wolfskill Orchard in Winters, California. Flies were reared on standard laboratory medium at room temperature. Virgins of both sexes were collected and aged for 3 d in vials (~20 flies per vial). On day 3, single females were aspirated (without CO_2) into individual vials. On day 4 at 8 a.m., 2 males were aspirated into each single-female vial for the courted and mated treatments. A third set of vials did not receive males. Vials were continuously monitored and for each female, if a mating occurred, the time was recorded at the start and end of mating. Males were removed upon completion of mating and males were also removed from a randomly selected vial in which a mating had not occurred. Thus, each mated female is "matched" by a female that had been courted for the same amount of time, but which had not mated. Trios of mated, virgin courted, and virgin unexposed females were simultaneously frozen in liquid nitrogen in separate tubes exactly 2 h after the completion of the mating. Thus, female age and time of day were precisely controlled. Flies were stored at –80 °C until RNA extraction. At the time of RNA extraction, each of the 3 treatments was separated into 3 replicate RNA preparations of 10 flies each to prepare probe for 9 chips.

Microarray sample preparation

Total RNA was extracted from whole flies using Trizol reagent (GIBCO, Carlsbad, Calif.). Affymetrix's Expression Analysis Technical Manual protocols were followed for cDNA synthesis (GIBCO SuperScript Choice System), cRNA processing and biotin-labeling (ENZO kit), and fragmenting. Oligonucleotide chips were probed, hybridized, stained, washed, and scanned at the UC Davis Core Facility according to Affymetrix guidelines.

Microarray data analysis

Expression data for 14 000 genes were retrieved using Microarray Suite 5.0 (MAS 5.0, Affymetrix) and dChip (Li

and Wong 2001, www.dchip.org). Each gene is represented by 14 pairs of 25-base oligonucleotide probes that either perfectly match the *D. melanogaster* reference sequence (PM) or mis-match the sequence at the central (13th) base of the probe (MM). MAS 5.0 generates a discrimination score for each probe pair. Probe pairs are then ranked using a Wilcoxon Signed Rank test and a *p* value is calculated for the probe set (i.e., gene) representing the confidence of a "present" vs. "absent" call for the gene. For our purposes, all genes were included in the analyses even if they were called absent in all 9 chips. However, only genes that showed fluorescence scores above 50 (dChip) or 100 (MAS) in at least 3 of the chips are considered in our sets of candidate genes. The signal calculation for a probe set was based on each data point measured relative to the median signal and weighted accordingly (One-Step Tukey's Biweight). For further information on the statistical algorithms, see www.affymetrix.com.

Li and Wong's dChip analysis was also used to generate expression values for each gene. dChip calculates model-based expression indices (MBEI) for both the PM-MM data (hereafter, dChipPM-MM) and PM-only data (hereafter, dChipPM). The MM probes are intended to serve as a control for transcriptional noise but may contribute more noise to the signal under certain circumstances (Li and Wong 2001; Irizarry et al. 2003). The dChip method gives different estimates of signal intensity than MAS 5.0. Instead of standard averaging, dChip pools replicate arrays and calculates an expression value for a particular gene across all replicates by down-weighting expression values with large standard errors. This is completed by a pooling and re-sampling method (see www.dchip.org). Overall chip signal intensities are normalized to the median intensity chip (#6 in this experiment), although results remain robust even when normalizing to the highest and lowest intensity chips.

We used Cyber-T to analyze the expression values generated by MAS 5.0 and dChip (http://visitor.ics.uci.edu/genex/cybert). Cyber-T uses a standard *t* test that incorporates prior information assuming that genes with similar signal intensities have similar measurement errors. This Bayesian estimate of within-treatment variation tends to reduce the rate of false positives in datasets without high replication. Additionally, by carrying out analyses on log-transformed data, the relationship between the mean and standard deviation is partially uncoupled and genes with lower mean expression values show higher standard deviations. This is biologically appropriate considering it is more difficult to accurately measure less abundant transcripts (Long et al. 2001). Parameters within Cyber-T were chosen as recommended by the authors (sliding window w = 101, Bayesian confidence value *r* = 10). Transcripts that showed the largest fold changes had very little statistical support suggesting simple ordering by fold-changes estimates is inappropriate to retrieve the best candidates (Baldi and Long 2001).

Cyber-T was used to identify possible cases of genes differentially expressed because of courting by comparison of data from unexposed virgins (3 chips) to that of courted virgins (3 chips). To detect genes differentially expressed after mating, data from the 3 replicate mated chips were compared with pooled data from the 3 replicate courted and 3

Fig. 1. Diagram of the number of genes showing significantly different ($p < 0.001$) levels of expression between virgin and mated females when using different data retrieval analyses. The fraction of genes confirmed by qPCR is also presented.
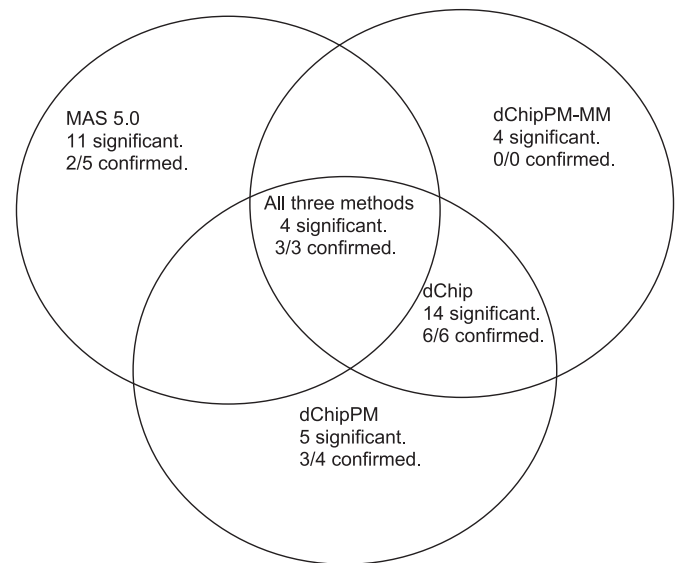
replicate unexposed virgin chips (6 chips). We arbitrarily chose a significance threshold of $p < 0.001$ to create a manageable number of candidates for quantitative real time reverse transcriptase PCR (hereafter, qPCR) quantification and other ongoing follow-up experiments. All data, including expression scores of every gene for each data extraction method as well as the Cyber-T analysis summary, are available upon request. Included in the supplementary tables are these data for the significant genes.

Real time quantitative reverse transcriptase PCR

We examined 18 genes using qPCR to investigate support for significant genes from the Affymetrix arrays using a different technology and to more precisely quantify expression differences between virgin and mated females. In a recent perspective outlining the pros and cons of confirming microarray data, the authors raised a concern that investigators might "cherry-pick" the genes to examine with a second technology such that confirmation is guaranteed (Rockett and Hellmann 2004). We avoid "cherry-picking" by examining the most significant genes from several different data retrieval methods. Total RNA was extracted from whole female flies subjected to the same protocol used to generate flies for the array experiments. Two independent RNA isolations were made for each of the mated (hereafter, M1 and M2) and virgin (V1 and V2) treatments (courted females were not examined in this experiment). RNAs were purified and DNased using the RNAeasy kit and Rnase-free Dnase set (Qiagen Inc., Valencia, Calif.). cDNA was made using first-strand Taqman RT reagents (Applied Biosystems, Foster City, Calif.). SYBR green PCR mix (ABI) was used for cDNA detection using primers designed on ABI's Primer Express software (primer sequences available upon request). Each reaction was run at a 25 μL volume. For each RNA prep/primer pair combination, 2 or 3 replicate SYBR green reactions were completed (e.g., on RNA prep M1, 2 separate

Table 1. Candidates with differential expression between unexposed virgin and courted virgin females ($p < 0.0005$).

Gene	Map	MAS 5.0		dChipPM-MM		dChipPM	
		p value	Fold	p value	Fold	p value	Fold
CG18628	67E5	9×10^{-6}	2.2	4.7×10^{-4}	1.8	1.7×10^{-4}	1.6
CG1620	43D1	2.1×10^{-4}	–2.2	4.2×10^{-4}	–2		
CG8628	65E7	1.1×10^{-4}	1.9				
CG15157	36F1	1.3×10^{-4}	6.9				
CG5817	35F9	1.5×10^{-4}	2				
Cyp6v1	19E1	2.7×10^{-4}	–2.8				
ShakB	19E3	2.1×10^{-4}	–2.3				
CG9772	82A4	3.2×10^{-4}	–1.9				
CG8329	67C3	3.8×10^{-4}	2				
CG14677	83C6	4.5×10^{-4}	5.4				
Smg	66F1	4.6×10^{-4}	–1.7				
CG31992	102D4					1.3×10^{-4}	–1.5
CG13602	95C13					2.3×10^{-4}	1.6
Ccp84Ac	84A3					2.4×10^{-4}	1.5
Kr-h1	26B8					2.9×10^{-4}	–1.7
Ubi-p63E	63F5					3.8×10^{-4}	–1.4
Arc70	102E5					4.1×10^{-4}	–1.5
CG2006	99B1					4.7×10^{-4}	1.5
CG12592	86B1			6×10^{-5}	–1.9		
CG13024	73D1			1.9×10^{-4}	2		
CG14190	18A3			2.3×10^{-4}	2.2		
CG31688	38C6			3.6×10^{-4}	2.1		
CG13134	31A2			4.2×10^{-6}	2.2		

Note: The p value and estimated fold change is represented for each of the datasets if the gene was significantly different at the $p < 0.0005$ level. Positive fold changes indicate higher expression in females exposed to courting males.

reactions (wells) were used to estimate the threshold cycle (C_t) for each gene. DNA contamination and primer-dimer were controlled for by carrying out 2 replicates of minus reverse transcriptase (-RT) reactions and 2 replicates of no template control (NTC). In the majority of -RT and NTC reactions, the C_t was "undetermined" at 50 cycles. We consider a C_t value >40 to indicate transcript absence. In the instances where there was an estimated C_t value for the -RT or NTC reactions, it was greater than a 4 cycle difference from C_t estimates of any RNA pool (the cut-off suggested in the ABI documentation) and no adjustments to the data were made. Additionally, melting curve analysis was used at the end of each run to verify product specificity. ABI SDS Version 2.1 software was used for visualization and quantification. Baseline and threshold values were appropriate at the software default levels and were not adjusted. To normalize variation in RNA abundances not related to our treatments, on every microtitre plate we ran housekeeping gene reactions on each RNA prep. Absolute amounts of RNA were fairly consistent across all RNA preps and microtitre plates (mean housekeeping gene C_t across plates = 23.8, standard deviation = 0.57). Estimates of transcript abundances for genes of interest were normalized to the estimate of the housekeeping gene on a per plate basis to account for the small variation in absolute amounts of RNA or pipetting error. This was accomplished by subtracting the housekeeping gene average C_t (across replicate wells) from the gene-of-interest average C_t for that prep (eg., RNA prep M1 *Gpdh* (housekeeping gene) C_t was subtracted from the same prep's C_t estimate of *CG18125* on that plate). This gave a normal-

ized relative C_t difference for the 4 preps (M1, M2, V1, and V2) for each gene. We then averaged M1 and M2 RNA preps together, subtracted this mated female average C_t from the average C_t of the V1 and V2 RNA preps, and used the delta-delta C_t method to estimate fold change (Livak and Schmittgen 2001). We present the average fold change detected using qPCR in the last column of Tables 2A and 2B.

Results

As detailed above, we used several different methods of microarray data extraction, MAS 5.0, dChipPM-MM and dChipPM, followed by data analysis in Cyber-T, to generate sets of candidate genes. We consider the best candidates to be those genes that were significant in all 3 data sets. Second tier candidates are those that were significant in 1 or 2 data sets. Figure 1 shows the number of significant ($p < 0.001$) genes detected in each analysis, as well as the number of genes positively confirmed by qPCR.

Courtship-induced changes of transcript abundance

To detect potential female-expressed genes influenced by the presence of courting males, we compared expression data from unexposed virgins (3 replicate chips) to courted virgins (3 replicate chips). We chose a higher significance threshold ($p < 0.0005$) for these comparisons than for the virgin vs. mated female comparisons because the smaller number of replicates could result in greater incidence of false-positives. The comparisons revealed only 1 gene, *CG18628*, which showed significantly different signal inten-

Table 2A. Transcripts detected in all analyses of Affymetrix chips as differentially expressed between virgin and mated females.

Gene	Map	MAS 5.0		dChipPM-MM		dChipPM		qPCR
		p value	Fold	p value	Fold	p value	Fold	Fold (avg.)
CG18125	35A4	3.7×10^{-5}	8.6	1.3×10^{-5}	28.4	2.1×10^{-12}	3.2	35.96
CG3036	25B1	3×10^{-6}	2.6	1.1×10^{-4}	1.7	1.2×10^{-5}	1.7	2.12
CG32834	59C1	2.3×10^{-5}	−2.3	4.7×10^{-4}	−1.7	7.9×10^{-5}	−1.5	−1.37
RpS6	7C2	6.8×10^{-4}	2.1	5.2×10^{-5}	2.2	1.3×10^{-4}	1.7	

Note: Positive fold changes indicate induced expression in mated females relative to the pooled set of virgin females. The last column indicates the average fold change as detected by qPCR.

Table 2B. Transcripts detected in 1 or 2 of the 3 analyses of Affymetrix chips as differentially expressed between virgin and mated females.

Gene	Map	MAS 5.0		dChipPM-MM		dChipPM		qPCR
		p value	Fold	p value	Fold	p value	Fold	Fold (avg.)
Try29F	29F7	3.8×10^{-6}	−4.3					−1.62
CG31218	87B9	5.9×10^{-5}	−4.5					(NC)
Ser8	50A8	2.2×10^{-4}	−1.7					(1.80)
CG12899	47A3	2.5×10^{-4}	−4.0					(NC)
CG6639	36C9	2.5×10^{-4}	−4.4					−3.18
CG6910	69A1			1.2×10^{-5}	1.9	5.7×10^{-4}	1.4	2.81*
CG31324	97A1			1.8×10^{-5}	2.4	1.4×10^{-6}	1.9	2.85
CG15096	55F5			8.3×10^{-4}	1.4	4.8×10^{-4}	1.4	1.48
CG16898	56F6			1.7×10^{-5}	−2.1	6.1×10^{-6}	−1.9	−2.03
fit	93F8			3.4×10^{-4}	1.5	8.8×10^{-4}	1.4	1.32
slif	80B2			1.1×10^{-4}	1.6	2.5×10^{-5}	1.5	2.39
cecB	99E2					6.6×10^{-5}	1.5	45.06
CG15721	11D5					5.3×10^{-4}	1.4	(−2.40)
CG17012	22D5					9.9×10^{-4}	1.4	1.39
CG5150	64D5					1.1×10^{-4}	1.5	3.68
CG3699	1D3	7.8×10^{-4}	−1.9					
CG7296	32A2	3.1×10^{-4}	2.0					
CG9571	19A6	9.1×10^{-4}	−5.2					
Ddc	37C1	6.4×10^{-4}	2.0					
CG32434	78A5	8.4×10^{-4}	−3.1					
Irk2	95A1	9.2×10^{-4}	−3.0					
Cp15	66D12			6.6×10^{-4}	1.3	8.6×10^{-4}	1.3	
Cyp28d1	25C10			6.6×10^{-4}	−1.4	5.7×10^{-4}	−1.6	
Cyp309a1	22F4			2.5×10^{-4}	−1.7	7.9×10^{-4}	−1.3	
CG6417	33E5			9.6×10^{-4}	−1.6	5.9×10^{-4}	−1.4	
CG14224	18E1			6.8×10^{-4}	1.7	5.0×10^{-4}	1.6	
Uro	28C3			1.1×10^{-4}	1.6	2.9×10^{-4}	1.6	
RpL12	60B7			3.3×10^{-4}	2.1	4.3×10^{-4}	1.5	
CG12726	11F8			4.3×10^{-4}	−1.4	4.2×10^{-4}	−1.4	
CG8083	45A1			9.6×10^{-4}	1.6			
Vanin-like	5E1			6.3×10^{-4}	1.7			
Reg-3	8D8			1.9×10^{-4}	1.6			
CG14248	97C3			3.4×10^{-4}	2.2			
Srp	89A13					3.9×10^{-4}	1.4	

Note: Positive fold changes indicate induced expression in mated females relative to the pooled set of virgin females. The last column indicates the average fold change as detected by qPCR. Numbers in parentheses indicate an estimate inconsistent between the array results and the qPCR results; and NC, means no change.

*, low confidence in fold estimate due to high C_t values.

sities across all analyses of Affymetrix data (Table 1, $p <$ 0.0005). An additional 22 genes showed differential expression between unexposed virgin and courted virgin females in only 1 or 2 of the analyses (Table 1). None of these courting-influenced candidates have been investigated by qPCR.

Mating-induced changes of transcript abundance

Comparison of gene expression data from virgin females (6 chips) vs. mated females (3 chips) using MAS 5.0, dChipPM-MM, and dChipPM generated 38 genes that showed significantly different signal intensities ($p < 0.001$) in virgin vs.

mated females (Tables 2A, 2B). However, only 4 of the 38 genes were significant across all 3 analyses (Table 2A). In comparison to other Affymetrix microarray experiments, 38 significant genes is a small number. One possible reason for this difference might be that we used a more stringent cut-off for determining significance. For example, in an investigation of fly immune response to microbial infection, DeGregorio et al. (2001) chose to consider 400 genes as significant in their experiment based on the overlap of their results with known immunity genes. While this is an intuitively satisfying approach, we have no a priori knowledge as to what types of genes might be influenced by mating, and therefore, such an approach is not possible here. In another Drosophila Affymetrix experiment, 127 genes were defined as age-regulated because they showed a greater than 1.8-fold change across 3 of 6 time points assayed (Zou et al. 2000). Our use of replicate microarrays and standard statistics such as the Bayesian t tests to identify the most strongly supported candidates takes the biological variability of some genes into account whereas fold-change based candidates often show limited statistical support (e.g., Pritchard et al. 2001; Baldi and Long 2001). Therefore, our statistical requirements might result in a smaller set of candidate genes. Alternatively, the smaller number of genes detected in our experiment might be due to the time point we chose to analyze for post-mating transcriptional differences. We chose 2 h after mating specifically to enhance for genes responding to seminal fluid transfer rather than related to the full onset of oogenesis. A later time point might detect more mating-influenced candidate genes.

qPCR analysis of array-based mating-influenced candidates

We used qPCR to independently investigate gene expression in 3 of the 4 genes that were significant in all 3 analyses of the array data, *CG3036*, *CG18125*, and *CG32834*. Our qPCR data for *CG3036* revealed a 2-fold increase of transcript post-mating, consistent with the results from the Affymetrix arrays (Table 2A). Affymetrix data for *CG18125* indicated absence of transcript in virgin females and low expression in mated females. However, qPCR data suggest that mating resulted in a 36-fold increase of *CG18125* transcript abundance. qPCR results for *CG32834* (formerly *CG9898*) are consistent with a small decrease in transcript abundance in mated females. The fourth gene, a ribosomal protein named *RpS6*, was significantly differentially transcribed in all 3 analyses but not examined with qPCR. Female specific ribosomal proteins have been detected in previous array work (Arbeitman et al. 2002). However, *RpS6* appears to be expressed in both sexes and to function in tumor suppression (see Flybase references).

The remaining 34 candidates were significant in either the MAS 5.0 data set or in 1 or both of the dChip data sets (Table 2B). qPCR data were collected for 6 of the 14 genes significant in both dChip analyses, 5 of the 11 genes significant only in the MAS 5.0 dataset, and 4 of the 5 genes significant in only the dChipPM analysis (Fig. 1).

The 5 genes selected from the MAS 5.0 analysis were *Try29F*, *CG31218* (formerly *CG17756*), *Ser8*, *CG12899*, and *CG6639*. Although all 5 genes showed significantly lower expression in mated females in the MAS 5.0 dataset, 3

showed no convincing evidence of reduced postcopulatory transcript abundance using qPCR. *CG31218* and *CG12899* showed no difference between virgin and mated females, and *Ser8* actually showed induced expression in mated females contrary to microarray results. *Try29F* was down-regulated in both the array and the qPCR, however, the latter assay suggests a less dramatic reduction in transcript in mated females. *CG6639*, which showed a roughly 4-fold reduction of transcript in mated females than virgins, had qPCR expression differences consistent with those detected on the Affymetrix chips. In summary, only 2 of 5 significant genes investigated from the MAS 5.0 analysis yielded qPCR results that were consistent with results from the microarray experiment.

Fourteen genes were significant in both the dChipPM and dChipPM-MM analyses. Of the 6 candidates from this group investigated by qPCR, all gave results consistent with expectations from the array experiments (Table 2B). The up-regulated chip-based candidates, *CG15096*, *slif* (*slimfast*), *fit* (*female-independent-of-transformer*), and *CG31324* (formerly *CG14557*) showed higher levels of transcript in mated females as estimated by qPCR. *CG6910* qPCR results were consistent with up-regulation, but C_t values were too high to be confident in the fold change. *CG16898*, down in mated females on the arrays, showed similar down-regulation in the qPCR assay.

We also examined 4 of the 5 genes that were significant only in the dChipPM data set. *CG17012*, which showed a significant 1.4-fold increase in transcript abundance post-mating in the Affymetrix experiment, showed a similar fold change in the qPCR data between virgin and mated females. *CecB* and *CG5150*, showed significantly higher expression in mated females, also consistent with the dChipPM data. *CecB* data shows a very large fold underestimation by the chip. Fold underestimates have been detected for both dChip and MAS methods previously and may be due to chip saturation or cross-hybridization (Rajeevan et al. 2001; Rajagopalan 2003; Rockett and Hellman 2004). *CG15721* showed increased transcript abundance in mated females in the Affymetrix data but was down-regulated in mated females in qPCR experiments, providing the only inconsistent result among the dChip candidate genes.

Discussion

Analyzing arrays

A major goal of whole-genome profiles of transcription is to identify genes or pathways previously unsuspected of playing a role in generating particular phenotypes. Our experiments provide the first genomic description of post-mating gene expression in *Drosophila melanogaster* females. To produce a solid list of candidate genes for further exploration, we pursued a strategy of generating candidate gene sets using several different analytical methods followed by qPCR confirmation of individual candidates. We note a few general patterns from comparison of these methods. If one were to assume that qPCR more accurately reflects expression levels than do expression arrays, our experiments suggest that data retrieved using dChip may provide more robust estimates of Drosophila transcript abundance compared with data retrieved using MAS 5.0. The genes de-

Table 3. EST (expressed sequence tag) and functional information on candidates that show transcriptional changes in females exposed to courting males.

Gene	Map	Functional activity	ESTs
CG18628	67E5	?	h, e
CG1620	43D1	ATP dependent helicase	h, e
CG8628	65E7	diazepam binding inhibitor	
CG15157	36F1	?	
CG5817	35F9	uncertain gene, transmembrane receptor	
Cyp6v1	19E1	cytochrome P450	
ShakB	19E3	shaking B (jump response)	
CG9772	82A4	ubiquitin dependent protein catabolism	
CG8329	67C3	chymotrypsin	
CG14677	83C6	immunoglobulin like	
Smg	66F1	negative regulation of translation	
CG31992	102C2	cell-surface receptor linked signal transduction	e, t, l
CG13602	95C13	?	t
Ccp84Ac	84A3	structural constituent of larval cuticle	e
Kr-h1	26B8	transcription factor	e, l
Ubi-p63E	63F5	protein degradation tagging	many
Arc70	102E5	RNA pol II transcription mediator	e, l
CG2006	99B1	?	
CG12592	86B1	?	
CG13024	73D1	involved in development	
CG14190	18A3	involved in transcription	
CG31688	38C6	?	
CG13134	31A2	cell adhesion, communication	

Note: The ESTs column indicates whether ESTs have been detected for the gene and where (e, embryo; h, head; l, larvae; and t, testes).

tected as differentially transcribed by all 3 methods or just both dChip methods were our overall "best" set of candidates, with all array candidates successfully validated in qPCR experiments. We did not complete qPCR on enough candidates that were significant in only 1 of the 2 dChip analyses (dChipPM vs. dChipPM-MM) to have any insight into which method may be superior. However, it is worth noting that we would have missed the interesting major induction of *CecropinB* in mated females if we had not completed the dChipPM analysis separately.

Precedent exists for a lower qPCR based confirmation rate on microarray candidates than might be expected although there may also be a bias in the literature against reporting genes that do not show patterns consistent with array results upon a second method of analysis (Rockett and Hellmann 2004). Rajeevan et al. (2001) confirmed 71% of their high density filter array candidates using qPCR (SYBR) and noted that the genes least likely to be confirmed are those with overall lower intensities and fold differences. Similarly, Wurmbach et al. (2003) confirmed 100% of their array candidates showing a >1.6-fold change with qPCR and only 66% of genes showing fold changes between 1.3 and 1.6-fold. However, our results suggest that the MAS 5.0 analysis performs poorly as the fold changes for the MAS candidates were greater on average than dChip candidate fold changes, yet the confirmation rate of MAS candidates was poorer.

A previous report examining the Affymetrix human Latin Square data set used several different methods of analysis, including MAS 5.0 and dChip (Rajagopalan 2003). Rajagopalan found MAS 5.0 performed better than dChipPM-MM, and

that dChipPM performed poorly (2003). The apparent discrepancy between those results and ours may be due to the fact that we used dChip and MAS 5.0 to retrieve the expression data but performed the statistical analyses using Cyber-T. Because we have no way of objectively determining which methods provide the best estimates of significant changes of transcript abundance, our discussion of candidate genes includes all 38 genes.

Insights into female postcopulatory molecular biology

Genes influenced by the presence of courting males

Our analysis of transcriptional changes in females associated with the presence of courting males revealed 23 candidate genes. *CG18628*, the most significant gene in the courting analysis, is induced in courted females. However, the fact that it has no sequence similarity to any known gene precludes speculation on its function. Genes significant in only one of the datasets include 2 proteins involved in protein degradation; *Ubi-P63E* and *CG9772* are both downregulated in courted females. Previous work has shown that Courtless, a ubiquitin-conjugating enzyme also involved in protein degradation, has important effects on male courtship behavior (Orgad et al. 2000). Mutant males with an excess of this protein fail to court almost entirely. Further work is necessary to determine whether the protein degradation pathway is generally important in courtship. Another candidate from our experiments, *ShakingB*, is a gene important in the jump response (Phelan et al. 1996). *ShakingB* shows reduced transcript abundance in courted females. It is tempting to

Table 4. EST and functional information on candidates that show postmating transcriptional changes.

Gene	Map	Activity	ESTs	DIRG?	Fold
CG18125	35A4	serine type endopeptidase			36
CG3036	25B1	sodium:phosphate symporter	e, l, t		2
CG32834	59C1	serine type peptidase	h		1.4
RpS6	7C2	ribsomal protein	h, l		2
Try29F	29F7	trypsin			1.6
CG31218	87B9	metallopeptidase			(NC)
Ser8	50A8	trypsin		Down (bact)	(1.8)
CG12899	47A3	?			(NC)
CG6639	36C9	serine type endopeptidase	e, l	Up (both)	−3.2
CG6910	69A1	oxidoreductase activity	h	Down (bact)	2.8
CG31324	97A1	cell communication, signal transduction			2.9
CG15096	55F5	inorganic phosphate:sodium symporter	h, l, t	Down (bact)	1.5
CG16898	56F6	?	t	Down (bact)	−2
fit	93F8	female-specific independent of transformer	h	Down (both)	1.3
slif	80B2	slimfast; cationic amino acid transporter			2.4
cecB	99E2	antibacterial peptide		Up (bact)	45
CG15721	11D5	kazal type serine protease inhibitor domain			(−2.4)
CG17012	22D5	serine type peptidase	h		1.4
CG5150	64D5	alkaline phosphatase	e, h		3.7
CG3699	1D3	oxidoreductase (detoxification)		Down (bact)	−1.9
CG7296	32A2	?	e, h, l	Up (bact)	2
CG9571	19A6	transcription factor			−5.2
Ddc	37C1	aromatic-L-amino acid decarboxylase	e, h	Up (fungal)	2
CG32434	78A5	loner; guanyl-nucleotide exchange factor			−3.1
Irk2	95A1	inwardly rectifying protein	e, h		−3
Cp15	66D12	structural constituent of chorion			1.3
Cyp28d1	25C10	cytochrome P450	e, h	Down (bact)	−1.5
Cyp309a1	22F4	cytochrome P450	e		−1.5
CG6417	33E5	sodium independent organic anion transporter	e		−1.5
CG14224	18E1	ubiquitin like domain	e, h, l		1.7
Uro	28C3	Urate oxidase		Up (both)	1.6
RpL12	60B7	structural constituent of ribosome	e, h		1.8
CG12726	11F8	defense response; chitin binding domain		immunity	−1.4
CG8083	45A1	nucleoside sodium:symporter			1.6
Vanin-like	5E1	hydrolase, pantetheinase			1.7
Reg-3	8D8	dihydropyrimidine dehydrogenase			1.6
CG14248	97C3	?			2.2
Srp	89A13	RNA polymerase II transcription factor	e		1.4

Note: The ESTs column indicates whether ESTs have been detected for the gene and where (e, embryo; h, head; l, larvae; and t, testes). The DIRG (Drosophila immune related gene) column indicates if the gene was detected to be induced, or down-regulated by fungal and (or) bacterial infection. The last column indicates the fold change (mated vs. virgin) in this experiment. If the gene was assayed using qPCR, then that value is presented, otherwise, it is the microarray average fold difference.

speculate that suppression of the female jump response could facilitate successful mating. This could be a female mediated mechanism ensuring successful copulation or a potential example of male manipulation of female behavior. Functions of the remaining "courtship" candidate genes are listed in Table 3.

Genes influenced by mating

A potentially important generalization from our experiment is the overlap of genes involved in the female-mating response and the response to microbial infection. Of the 14 000 genes represented on the Affymetrix GeneChip, 400 were identified as Drosophila immunity related genes (DIRGs -DeGregorio et al. 2001). Within our set of 38 mating-affected candidate genes, 12 are considered DIRGs, a clear excess over the number expected by chance (Fisher's exact test, $p < 10^{-7}$, De Gregorio et al. 2001). This is unlikely to be a spurious result from bias in the arrays as none of our 23 courting-affected candidate genes overlapped with the set of DIRGs (Fisher's exact test, $p < 0.007$). Given that the experiments from De Gregorio et al. (2001) used only males, while our experiments were on females, the genes in common are unlikely to be sex-specific in expression. Determining whether or not a greater fraction of our mating-influenced candidates would overlap with female immune-related genes awaits microarray experiments on females subjected to microbial infections. Several immune related genes that are candidates in our experiments are particularly intriguing. The antimicrobial *CecropinB* is highly induced in mated females. *CecropinB* is constitutively expressed in the

spermathecae and the seminal receptacle (Tzou et al. 2000). Induction of antimicrobial peptides in mated females could be a response to sexually transmitted bacteria or could serve to facilitate successful long-term sperm storage. We are carrying out more detailed characterization of temporal and spatial patterns of mating-induced *CecB* to illuminate its potential role in reproduction. *CG6639*, the most highly induced gene upon bacterial infection in two separate array studies, is down-regulated in mated females in our experiments (DeGregorio et al. 2001; Irving et al. 2001). In a recent screen for sex-biased gene expression in the head, *CG17820* was discovered to show expression in the fat cells of the female head (Fujii and Amrein 2002). The function of this gene, now named *fit* (female-specific independent of transformer) has not yet been determined, however, it appears to be induced in mated females in our experiments and down-regulated in infected males, perhaps indicating variation in sex-specifity (De Gregorio et al. 2001). At least 9 other immune-related genes significant in our experiment are noted in Table 4. The overlap of genes responding to infection in males and mating in females suggests the possibility of interesting connections between immunity and sex. For example, mating-induced changes of immune related gene transcript abundance may reflect microbial infection, trade-offs between immunity and reproduction, or even direct interactions between females and male-derived proteins transferred during mating.

Over 200 serine proteases are present in the Drosophila genome (Ross et al. 2003). More serine proteases are influenced by mating ($n = 6$) than expected by chance (Fisher's exact test $p < 10^{-4}$). This test is somewhat conservative because not all serine proteases are represented on the Affymetrix microarray. The notion that serine proteases contribute to female postcopulatory responses is plausible given previously collected data, and is particularly interesting due to the potential for direct male-female molecular interactions. For example, serine proteases and serine protease inhibitors (serpins) are present in seminal fluid (Coleman et al. 1995; Swanson et al. 2001). Male expressed serpins may function to protect Acps from premature cleavage by proteases present in the seminal fluid. Acp cleavage upon transfer to females is not uncommon. Both *Acp36DE* and *ovulin* are cleaved upon transfer to females, with cleavage requiring components from both sexes (Monsma et al. 1990; Park and Wolfner 1995; Bertram et al. 1996). Male-derived serpins could also function to prevent female proteases from modifying Acps (Wolfner 2002). Thus, the presence of proteases and serpins in seminal fluid and in female postcopulatory responses could represent a struggle for control over certain aspects of reproduction. The evolutionary plausibility of this notion has been demonstrated in several experiments that suggest that the reproductive interests of male and female *D. melanogaster* are not always concordant (Rice 1996; Holland and Rice 1999). Much more data on the functional biology of these molecules is necessary for generating clear evolutionary hypotheses.

Sodium-phosphate symporters represent another functional category of genes that may play a role in post-mating responses. *CG3036* and *CG15096* showed induced transcription in mated females in our experiments. Twenty-one sodium:phosphate transporters exist in the Drosophila genome.

Thus, detecting 2 that are induced by mating is unlikely by chance (Fisher's exact test, $p < 0.002$). Previous experiments have shown that *D. melanogaster* females incorporate phosphorus derived from male seminal fluid into ovarian nucleic acids and mature eggs (Markow et al. 2001). The effect of male-derived phosphorous on female fitness is unclear. However, reduced dietary phosphorus hinders Drosophila oogenesis (T. Markow, pers. comm.), suggesting that female uptake of male-derived phosphorus could benefit females. Clearly, additional experiments would be required to determine whether CG3036 and CG15096 proteins specifically transport male-derived substances as opposed to endogenous substrates.

Results from the experiments discussed here motivate directed questions regarding the connection between immunity and sex, the possibility of male-derived donations of phosphorus, fitness consequences of such donations, and the involvement of serine proteases in Drosophila post-mating male-female interactions. Additionally, detailed investigation of specific candidate genes identified in the experiments described here are likely to reveal several insights into the postcopulatory biology of female *D. melanogaster*. However, it is important to note that microarray experiments in general are limited in the ability to detect genes of interest both due to the requirement for transcriptional change to identify candidates and due to the assumption that transcript levels show a strong correlation with protein levels. Other strategies, such as EST studies on female reproductive tissues, population genetic work on sex-related female-specific genes, and genetic analyses of female components of sperm storage and competition are necessary to gain a deeper understanding of the co-evolutionary path of the sexes.

Acknowledgements

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