# Mating-responsive genes in reproductive tissues of female *Drosophila melanogaster*

**Paul D. Mack*, Anat Kapelnikov†, Yael Heifetz†‡, and Michael Bender*‡**

*Department of Genetics, University of Georgia, Athens, GA 30602; and †Department of Entomology, Hebrew University of Jerusalem, Rehovot 76100, Israel

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**Male-derived accessory gland proteins that are transferred to females during mating have profound effects on female reproductive physiology including increased ovulation, mating inhibition, and effects on sperm utilization and storage. The extreme rates of evolution seen in accessory gland proteins may be driven by sperm competition and sexual conflict, processes that may ultimately drive complex interactions between female- and male-derived molecules and sperm. However, little is known of how gene expression in female reproductive tissues changes in response to the presence of male molecules and sperm. To characterize this response, we conducted parallel genomic and proteomic analyses of gene expression in the reproductive tract of 3-day-old unmated and mated female *Drosophila melanogaster*. Using DNA microarrays, we identified 539 transcripts that are differentially expressed in unmated vs. mated females and revealed a striking peak in differential expression at 6 h postmating and a marked shift from primarily down-regulated to primarily up-regulated transcripts within 3 h after mating. Combining two-dimensional gel electrophoresis and liquid chromatography mass spectrometry analyses, we identified 84 differentially expressed proteins at 3 h postmating, including proteins that appeared to undergo posttranslational modification. Together, our observations define transcriptional and translational response to mating within the female reproductive tract and suggest a bimodal model of postmating gene expression initially correlated with mating and the final stages of female reproductive tract maturation and later with the declining presence of male reproductive molecules and with sperm maintenance and utilization.**

accessory gland proteins | reproduction | reproductive tract | sperm | sexual conflict

For all internally reproducing organisms, the female reproductive tract must provide the appropriate environment in order for fertilization to succeed. In addition to direct gamete-gamete interactions, numerous other reproductive functions are integral to successful fertilization. In insects, processes such as sperm storage and maintenance and egg activation are likely to result from interactions between eggs, sperm, the female reproductive tract, and molecules contributed by both sexes (1–3). This phenomenon is best known from male insects; males of many species transfer seminal fluid proteins [primarily accessory gland proteins (Acps)] that influence female reproductive physiology. In *Drosophila*, for example, Acp36DE is transferred in the seminal fluid and is required for efficient sperm storage in the female sperm storage organs (4). *Drosophila* males produce reproductive molecules that influence a wide range of processes, including oogenesis, egg-laying, female receptivity, and sperm transfer and storage (2, 5), and which are also harmful to females (6). Females also secrete molecules from their reproductive epithelia (7) that may influence fertilization, yet we know little about the identity or function of these molecules.

Female reproductive molecules are likely to interact with male seminal fluid and sperm given that *Drosophila* sperm storage organs, the seminal receptacle and paired spermathecae, and the female accessory glands feature ducts that open onto the precise location where eggs are fertilized (8). Secretions from female reproductive epithelia may lubricate the reproductive tract, protect eggs after deposition, provide immune defense, or function in sperm maintenance, storage, and release (9, 10).

In *Drosophila*, physiological changes follow soon after mating. Ovulation and egg-laying begin at ≈90 and ≈180 min postmating (11). Sperm storage generally peaks at 1 h postmating after which competent sperm must be maintained (9). The capability to store sperm facilitates postmating competition between male ejaculates that may conflict with female reproductive interests (12). Because male and female fitness interests often differ, processes that determine which sperm fertilize eggs are likely to coevolve antagonistically and may influence the evolution of sperm competition and cryptic female choice (1).

Recent studies have made important contributions to our understanding of postmating gene expression in *Drosophila* females (13, 14). However, these studies were restricted to analysis of RNA expression at a single postmating time point in whole females. Here we have defined transcriptional and translational response to mating specifically within the lower reproductive tract. DNA microarray analysis identified 539 differentially expressed RNA transcripts within the lower reproductive tract of unmated vs. mated 3-day-old *Drosophila* females at 0, 3, 6, and 24 h postmating. This assay revealed a distinct shift from gene silencing to gene activation soon after mating and a dramatic peak in differential gene expression at 6 h postmating. In parallel, we identified 84 differentially expressed proteins at 3 h postmating via a combination of two-dimensional gel electrophoresis (2D-PAGE) and liquid chromatography mass spectrometry (LC-MS/MS); these proteins were primarily down-regulated and included proteins that appeared to undergo posttranslational modification. Our observations suggest a bimodal expression response to mating driven initially by mating and the presence of male seminal fluid components and subsequently by later postmating processes such as sperm maintenance and utilization.

## Results

**Mating Results in Gene Expression Changes in the Lower Reproductive Tract.** To identify genes whose expression changes after mating, we dissected the lower reproductive tract from unmated females and from mated females at 0, 3, 6, and 24 h postmating. During this period, the reproductive tract undergoes significant physiological changes to prepare for fertilization and the rapid and efficient use of sperm (9). The tissues dissected include the lower part of the common oviduct, the seminal receptacle, the paired female accessory glands and spermathecae, and the anterior uterus (Fig. 1*A*), which together comprise the site of fertilization and sperm storage. RNA was extracted from pooled tissues,

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Abbreviations: Acps, accessory gland proteins; GO, gene ontology; LC, liquid chromatography; 2D-PAGE, two-dimensional–PAGE.

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‡To whom correspondence may be addressed. E-mail: heifetz@agri.huji.ac.il or bender@uga.edu.

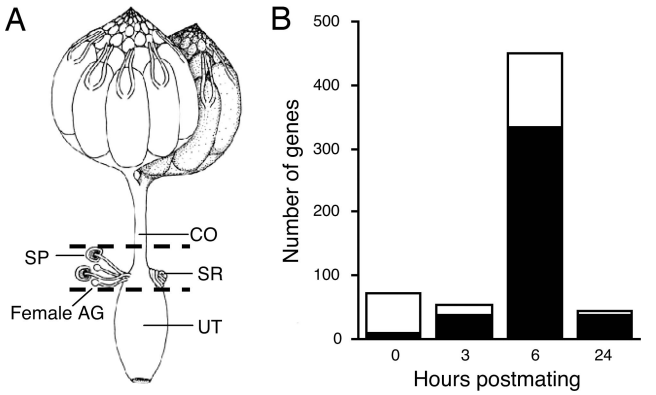


**Fig. 1.** Postmating gene expression changes in the lower reproductive tract. (*A*) Dotted lines depict the boundaries of tissue assayed. UT, uterus; SR, seminal receptacle; SP, spermatheca; female AG, female accessory gland; CO, common oviduct. Modified from ref. 9. (*B*) Whole bars indicate the number of differentially expressed genes ($P < 0.0001$) at each time point in mated 3-day-old females compared with unmated 3-day-old females. Black shading in each bar represents the proportion of genes up-regulated in mated females; white portion of bar represents the proportion of genes down-regulated in mated females.

labeled, and hybridized to Affymetrix (Santa Clara, CA) *Drosophila* Genome Arrays (See *Materials and Methods*). Array data were analyzed by using robust multichip average (RMA) analysis and a probe-based linear model (15) and RMA analysis (16). This analysis identified a total of 539 genes whose expression levels differed significantly ($P < 0.0001$) in the reproductive tract of unmated vs. mated females at 0, 3, 6, or 24 h postmating. Table 1 categorizes the differentially expressed genes into gene ontology (GO) functions, and a complete list of these genes is given in Table 3, which is published as supporting information on the PNAS web site. Among the 290 genes that are currently assigned GO terms, the largest categories are nucleic acid binding (103 genes), catalytic activity (70 genes), and transport activity (39 genes). Other categories of interest from the perspective of female mating response are defense response (25 genes), signal transducer (25 genes), and protease/peptidases (17 genes).

Fig. 1*B* shows that we identified 71 differentially expressed genes immediately after mating (0 h), and 55, 451, and 44 genes at 3, 6, and 24 h postmating, respectively. We observed a large peak of differentially expressed genes at 6 h postmating. Of the 539 genes represented in Fig. 1*B*, 381 were differentially expressed only at 6 h (305 up-regulated and 76 down-regulated).

**Table 1. GO of mating-responsive genes**

| Molecular function | No. of genes |
|---|---|
| Unclassified | 249 |
| Nucleic acid binding | 103 |
| Catalytic activity | 70 |
| Transport activity | 39 |
| Structural molecule | 27 |
| Defense response | 25 |
| Signal transducer | 25 |
| Transcription regulator | 23 |
| Protease/peptidase | 17 |
| Translation regulator | 10 |
| Chaperone activity | 9 |
| Antioxidant | 5 |
| Cell adhesion | 5 |
| Apoptosis regulator | 2 |
| Motor activity | 2 |

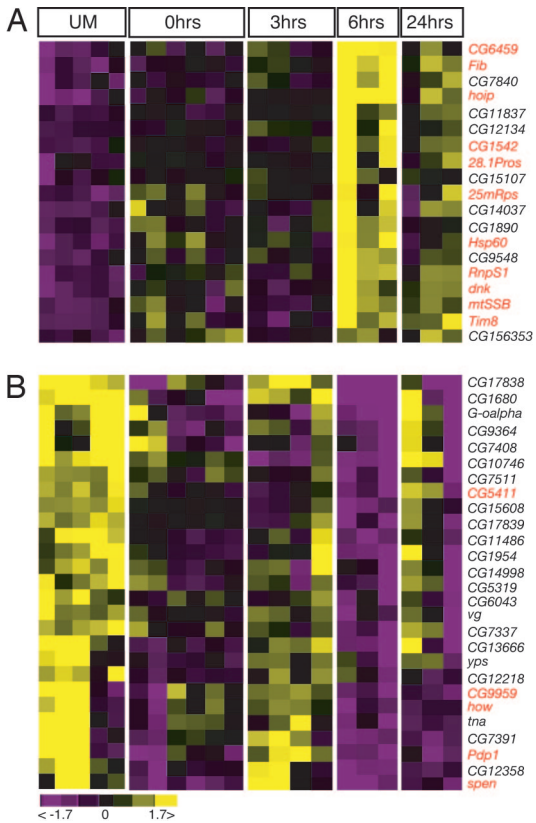


**Fig. 2.** Clustering of differentially expressed genes. (*A*) Genes up-regulated in mated females at 6 h postmating. Genes in the GO category protein complex are in red. (*B*) Genes down-regulated in mated females at 6 h postmating. Genes in the GO category mesoderm development are in red. Each column within each time point represents data from a single array. Yellow, increased expression; blue, decreased expression relative to normalized average expression for that gene among all arrays; black, no change in expression.

Furthermore, of the 72 genes that exhibited differential expression at more than one time point, all but three exhibited peak expression changes at 6 h. Taken together, these observations emphasize the dramatic peak of gene expression activity at 6 h.

When we compared the mating-responsive genes we observed to those expressed in whole body assays (13, 14), we found that only 3% (59/1,783 and 1/38, respectively) of the genes identified in whole body assays were differentially expressed in the lower reproductive tract. Of these 59 genes (13), two (*CG16840* and *CG18067*) were mediated by Acps, 29 by sperm, and 28 by non-sperm, non-Acp effects (13). This pattern suggests that, at 3 h postmating, gene expression changes are primarily mediated by sperm or by non-sperm, non-Acp effects. Most of the overlapping genes exhibited more extreme changes than those observed in whole body assays, consistent with dilution by RNA from other body parts. Ease analysis (http://david.niaid.nih.gov/david/ease.htm) of this subset of genes showed that they are enriched for genes involved in immune response ($P = 0.0133$), protein targeting ($P = 0.0164$), and cell organization and biogenesis ($P = 0.0271$). In addition, only 4.6% (23/500) of the genes previously identified in an expressed sequence tag (EST) study of the female reproductive tract (17) overlapped with the mating-responsive genes identified in our study.

To further characterize the peak response, we used PCluster, a clustering tool within the program SCOREGENES (see *Materials and Methods*), to identify significantly differentially expressed genes at 6 h that had similar temporal expression patterns. Fig. 2 shows the two clusters that exhibited the greatest differences

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in expression. Fig. 2*A* depicts genes up-regulated in mated females at 6 h. This cluster was enriched for genes involved in both primary metabolism ($P = 0.00029$) and protein complexes ($P = 0.035$), including *Pros28.1*, a subunit of the proteasome that catabolizes cellular proteins and the mRNA-binding protein, *S1* (*RnpS1*); genes in the protein complex category are highlighted in Fig. 2*A*. Several mitochondrial proteins, such as *mitochondrial ribosomal protein S25* (*mRpS25*) and *heat shock protein 60* (*Hsp60*), were also present in this cluster; such genes may function to provide the energy required to produce and modify the products of the many genes up-regulated during this period. Interestingly, Hsp60 is found on the luminal surface of bovine oviductal epithelia where it binds, and presumably maintains, sperm (18).

A second cluster of genes down-regulated in mated females at 6 h postmating (Fig. 2*B*) was enriched for genes involved in mesoderm development ($P = 0.0025$) and transcription ($P = 0.0017$); genes in the mesoderm development category are highlighted in Fig. 2*B*. The cluster includes such genes as the polyA-binding protein, *pdp1*, and the somatic muscle development gene, *held out wings* (*how*), both of which are involved in muscle development. *Protein C kinase 98E* (*Pkc98E*) and *G-oα47A* are involved with neural development and axon guidance and are in flux at a time when interaction between nerve tissue innervating the reproductive tract appears to be very important (19). Further analysis will be necessary to assess how the down-regulation of these genes influences reproductive physiology.

Our genomic assays also revealed a clear shift from primarily down-regulated genes immediately after mating (84.5%; 60/71 genes at 0 h) to primarily up-regulated genes at all other time points (minimum 67%; Fig. 1). Two genes that function in exocytosis and endocytosis (*Rab10* and *tomosyn*), one with transmembrane receptor activity (*Nrx*) and one with a predicted role in hormone secretion (*CG9066*), were observed in the down-regulated group at 0 h; these four genes were among 23 genes that were down-regulated at 0 h but not differentially expressed at any other time. This observed shift from down-regulated to activated genes parallels the female's shift from copulation to sperm storage, fertilization, and sustained reproduction.

**Mating Induces Down-Regulation of Proteins in the Lower Reproductive Tract.** To identify proteins that undergo changes in expression level in the lower reproductive tract in response to mating, we conducted 2D-PAGE in parallel with our transcriptome assays. Here we compared expression levels in the lower reproductive tract between unmated and mated females at 3 h postmating. We visualized 341 spots from unmated and 325 from mated female lower reproductive tracts (Fig. 3 *A* and *B*), identifying 26 protein spots that were differentially expressed in mated females. MS identification indicated that these 26 spots contained 63 identifiable proteins, including 9 that were identically differentially expressed in at least two separate biological replicates (Table 2). Five of the nine proteins were up-regulated after mating including one, Upheld (also known as troponin-T), which increased >8-fold in mated females. Upheld is the key component in regulating the tropomyosin-troponin complex and thus plays an essential role in muscle contraction (20). Two of these nine proteins were down-regulated in mated females (CG9391, inositol-1(or 4)-monophosphatase activity; CG6439, isocitrate dehydrogenase [NAD+] activity). The remaining two proteins, glycogen phosphorylase and translationally controlled tumor protein, exhibited a concurrent increase and decrease of adjacent spots in mated females relative to unmated females (Table 2). These reciprocal changes indicate probable isoform differences because of posttranslational modification or alternative splicing. Thus, mating appears to induce further modification of proteins that may play important physiological roles in female postmating biology.

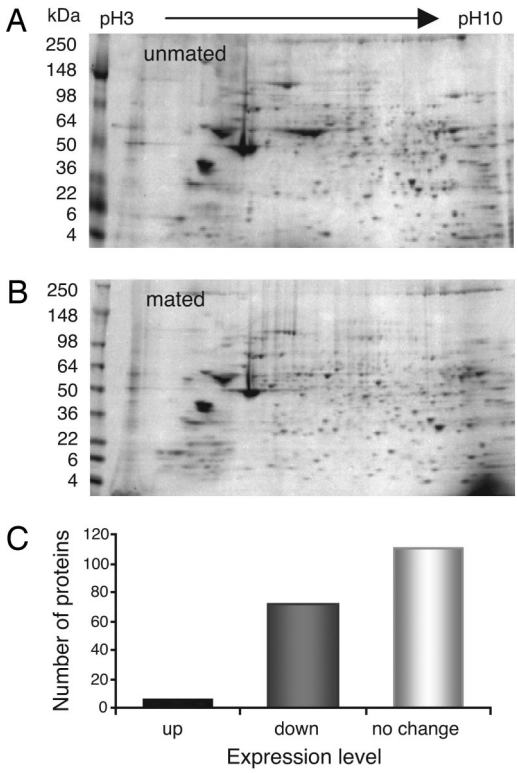


**Fig. 3.** Mating induces changes in the reproductive tract protein expression profile. Reproductive tracts of unmated (*A*) and mated (*B*) females at 3 h postmating. The approximate isoelectric points (pI) and molecular masses (kDa) of the proteins are indicated at the top and left, respectively. (*C*) Proteins identified in the lower reproductive tract of unmated and mated females at 3 h postmating determined by LC-MS/MS are categorized as up-regulated in mated females (up), down-regulated in mated females (down), or unchanged.

To obtain a more thorough catalog of mating-responsive proteins, we compared mated females dissected at 3 h postmating with unmated females by using LC-MS/MS. We identified an additional 175 proteins (see Table 4, which is published as supporting information on the PNAS web site) by using this method of which 3% (5/175) were up-regulated and 41% (72/175) were down-regulated in mated females (Fig. 3*C*). The remaining 98 proteins were present in unmated and mated female tissues at equivalent levels. Two up-regulated proteins were not detected in unmated females, including transaldolase, an important enzyme in the metabolic network that controls oxidative stress (21), that may also be involved in ecdysteroid synthesis, because molt-inhibiting hormone, a neuropeptide that controls ecdysteroid biosynthesis, has been shown to regulate transaldolase (22). Two glycolytic enzymes needed for muscle function were also up-regulated in mated females. One, aldolase, localizes along the sarcomere and is involved in a mechanism by which ATP is supplied to myosin ATPase (23). Limpet, a protein with transcription factor activity that may also be involved in muscle development, was also up-regulated. In contrast, down-regulated proteins consisted largely of proteins involved in functions such as protein biosynthesis (e.g., ribosomal protein L23 and ribosomal protein L4), ATP-binding proteins (e.g., Vha68–2 and heat shock protein 83), actin binding (e.g., tropomyosin 1, Chd64, and CG5023), and general metabolism (e.g., pyruvate dehydrogenase and pyruvate kinase). Nineteen of the down-regulated proteins were detected only in the lower repro-

**Table 2. Quantitative changes in protein expression (2D-PAGE)**

| FBgn | Protein name | GO: molecular function | Fold change | Score |
|---|---|---|---|---|
| FBgn0004169 | Upheld (Troponin T) | Tropomyosin binding | 8.45 | P |
| FBgn0032237 | | L-malate dehydrogenase activity | 1.93 | P |
| FBgn0034432 | | Very-long-chain-acyl-CoA dehydrogenase activity | 3.14 | P |
| FBgn0005585 | Calreticulin | Calcium ion binding | 1.31 | P |
| FBgn0027657 | Globin 1 | Oxygen transporter activity | 1.61 | P |
| FBgn0037063 | IMP | Inositol-1(or 4)-monophosphatase activity | −2.32 | P |
| FBgn0038922 | | Isocitrate dehydrogenase (NAD+) activity | −3.79 | I |
| FBgn0004507 | Glycogen phosphorylase | Glycogen phosphorylase activity | PTM | P |
| FBgn0037874 | Translationally controlled tumor protein | DNA binding | PTM | P |

PTM, post translational modification; P, positive; I, intermediate.

ductive tracts of unmated females (e.g., Rtnl1- receptor signaling protein activity).

In sum, we identified 10 up-regulated, 72 down-regulated (the two down-regulated proteins identified by 2D-PAGE were also identified by LC-MS/MS), and 2 translationally modified proteins by using the two different methods. Our observation that many more proteins are down-regulated than are up-regulated may suggest that the majority of proteins required for the early stages of postmating reproductive physiology are synthesized before mating and that females may be shifting from a posteclosion developmental phase during which they reach reproductive maturity to one in which they prepare for maximum reproduction. This observation is consistent with a model proposing that the female reproductive tract is poised for production of large numbers of eggs but is arrested until mating (13, 19). In this model, newly synthesized proteins might include, for example, those components required to maintain sperm function until they are needed for fertilization.

**Comparison of Genomic and Proteomic Data Sets.** To examine whether changes in the profile of protein expression were correlated to changes in transcript expression in the lower reproductive tracts of mated females, we categorized the 10 up-regulated and 72 down-regulated proteins obtained from the combined 2D-PAGE and LC-MS/MS data sets. We first examined up- and down-regulated proteins to determine presence or absence of transcripts in unmated and mated females. Proteins with transcript found at all time points were classified as positive, those with transcripts observed at no time points were classified as negative, and all others were classified as intermediate. Eight of the 10 up-regulated proteins were categorized as positive, and 2 were categorized as negative. Of the 72 down-regulated proteins, 59 were positive, 9 negative, and 4 intermediate. Thus, we found transcripts in both unmated and mated females throughout the assay period for the majority (74/84; includes the two translationally modified proteins) of the differentially expressed proteins. However, none of the positive or intermediate proteins exhibited corresponding up- or down-regulation in transcript levels.

We observed only a small number of mating responsive proteins that were also identified among the transcriptome in whole body assays of mated females [52/1,783 (13) and 0/38 (14)]. Sixty-seven percent (35/52) of these transcripts were regulated by sperm, and 10% (5/52) were regulated by Acps; none appear to be highly expressed (>1.5-fold) in whole-body assays. A slightly greater fraction of our mating responsive proteins (6.2%; 32/500) were identified in an EST screen of reproductive tracts from female *Drosophila* of various ages and mating histories (17). This lack of correspondence in expression pattern suggests that proteomic and genomic techniques may be identifying complementary sets of gene products and indicates the value of using both simultaneously.

Comparing the two data sets also enabled us to identify genes that may be components in the same or related pathways. For example, the transcript for juvenile hormone epoxide hydrolase 2 (jheh2) was down-regulated in females dissected at 6 h postmating and Vha44 (vacuolar H+ ATPase 44kD C subunit), a gene involved in the regulation of juvenile hormone (JH) biosynthesis, was differentially regulated at 3 h postmating. In the proteomic analysis, the protein CG10527 was identified with predicted farnesoic acid *O*-methyltransferase activity based on sequence similarity. Farnesoic acid *O*-methyltransferase mediates the methylation of farnesoic acid to methyl farnesoate. This observation suggests that pathways associated with the synthesis or degradation of JH, a hormone previously shown to play a role in mating and reproduction for both males and females in a diverse array of arthropod taxa (24), are active in the reproductive tract and are affected by mating.

## Discussion

We used DNA microarrays to identify 539 genes that are differentially expressed in the lower reproductive tracts of unmated vs. mated females at a time when several critical physiological changes occur in females. Our analysis revealed a pronounced peak of differentially expressed genes at 6 h postmating, after sperm storage is complete and Acps, with the exception of sex peptide, are no longer detectable (25, 26). This expression peak at 6 h postmating, primarily of genes up-regulated in mated females, also coincides with the greatly increased reproductive activity that follow mating (11, 27). In contrast, most differentially expressed genes observed at 0 h postmating and most proteins expressed at 3 h postmating were down-regulated in mated females.

By integrating parallel gene and protein expression technologies, we obtained a more comprehensive assessment of changes induced by mating in the lower reproductive tract than would be possible by either technology alone. First, our observation of corresponding transcripts for the majority of proteins identified provides a degree of validation for the two data sets. Second, our detection of differentially expressed proteins without corresponding differentially expressed transcripts suggests that mRNA and protein expression levels may not be strictly coupled. Previous comparisons of proteomic and genomic assays have found only weak to intermediate correlations between protein and mRNA abundance, especially among low abundance proteins (28, 29). Thus, although we cannot exclude experimental error as discussed in ref. 30, our observations suggest that some postmating changes in expression may be mediated by translation initiation or by posttranslational mechanisms such as targeted protein degradation (31). Third, our observation of proteins that were differentially expressed in the apparent absence

of corresponding transcript may suggest rapid posttranslational degradation of transcript. Maziarz *et al.* (30) found that 10% of proteins identified in mouse islet cells had no corresponding transcript, similar to the 12% we observed in our study. If these differences are not simply due to the limits of detection of microarray hybridization protocols, they merit additional examination because such proteins may represent factors important in regulating specific biological functions essential for postmating events, such as sperm storage and fertilization. Within the limits of current DNA microarray technology, we believe we have detected the majority of postmating transcription changes in the lower reproductive tract after mating. However, the lack of more complete overlap between genomic and proteomic data sets and limitations in the sensitivity of proteomic assays suggest that it is unlikely that we have identified the majority of proteins present in these tissues after mating.

Our observation of differentially expressed genes involved in muscle activity may indicate a suite of genes influencing muscular activity in the *Drosophila* female reproductive tract. Upheld, detected in both of our proteomic assays, is the principle link in the regulation of key components of the tropomyosin-troponin complex that mediates muscle contraction (20). Mated females also reduced transcript expression for *pdp1* and *how*, both involved in mesoderm development (32, 33). We speculate that down-regulation of these genes is indicative of the completion of muscle maturation or differentiation processes at 6 h postmating. Muscle tissue is integrated throughout the *Drosophila* female reproductive tract, and muscular contractions have been implicated in female-mediated sperm storage and displacement processes in a host of insect taxa, including *Drosophila* (34–36). Furthermore, both the seminal receptacle and the ducts of the spermathecae have muscle layers and sphincters (8, 37).

Our observations of an initial down-regulation of gene expression followed by peak expression change at 6 h postmating suggest that there may be two waves of transcriptional regulation relative to a female's first mating opportunity: one shortly after mating and a second ≈6 h after mating occurs. Newly eclosed female *Drosophila* typically undergo a 3-day period of reproductive maturation during which the reproductive tract becomes "poised for mating." After mating, females complete development of the reproductive tract primed for efficient fertilization (19). We hypothesize here a second switch point occurring at ≈6 h postmating to sustain elevated fertilization and reproduction, influenced by the female's regulatory and physiologic responses to seminal fluid components and newly stored sperm. Many protein components required for optimal reproduction may be produced by unmated females during premating reproductive maturation (13, 19). The small number of proteins we identified, the relative paucity of differentially expressed transcripts before 6 h postmating, and the limited number of genes that exhibited differential expression in excess of two-fold appear to fit with this model and are also consistent with prior studies in whole mated females (13, 14).

As female reproductive molecules are identified and as their functions are better understood, it will be interesting to discern the dynamics of their interactions with male reproductive molecules. When evolutionary changes confer fitness benefits to one sex at a cost to the other, male and female processes that contribute to reproduction may coevolve antagonistically (38, 39). A greater understanding of how females respond genetically and physiologically to mating will help us better understand the molecular aspects of sperm competition (40), sexual conflict (39), and cryptic female choice (1).

## Materials and Methods

**General Assay Methods.** Males and females were created by crossing two different pairs of four extraction lines isogenic for chromosomes II and III. The origin of these four unique lines (line numbers 112, 140, 234, and 348) is described elsewhere (41). One cross (234 male × 140 female) produced F1 females for mating; the other pair (112 male × 348 female) was used to produce males. F1 flies created as described were thus identically heterozygous for chromosomes II and III. All flies were kept in a 12-h light/dark cycle at 23 ± 2°C. Before mating, F1 females and males were collected on ice and then held separately until 3 days of age. At the end of copulation, females were separated from males, and lower reproductive tract tissues (Fig. 1*A*) were dissected at the appropriate postmating time. At least three independent biological samples were created for each treatment and time point for both the genomic and proteomic assays.

**Microarray Assays.** Tissues were stored in TRIzol (Life Technologies) plus 0.5X Yamamoto's Ringer's solution and placed at −80°C. We pooled tissues from ≈40 females for each sample and extracted total RNA via a standard protocol (42). Each sample was assessed for RNA quality and concentration by spectrophotometry. Each sample was hybridized to oligonucleotide *Drosophila* Genome (GeneChip) arrays (Affymetrix, Santa Clara, CA). Labeling and hybridization were performed by the Molecular Biology Core Facility at the Medical College of Georgia.

**Microarray Data Analysis.** We used robust multichip average analysis (16), to simultaneously normalize data from all groups and a probe-based linear model that accounts for array effects (15) to identify genes with significant changes in expression between groups; each biologically independent sample was considered in the same way by this method. In addition, by using unsupervised hierarchical biclustering (43), we found that the expression profiles of replicates within the same time point grouped together (data not shown).

After excluding from our analysis any genes that were not found to be present by Affymetrix MAS 5.0 on at least one array from either the unmated or mated female groups, 8,280 genes remained. We indirectly controlled for multiple tests by setting the critical $P$ value at 0.0001, which yielded a false positive discovery expectation of less than one gene (8,280 × 0.0001 = 0.83 false positives expected) for the entire genome.

For the clustering analysis, we used SCOREGENES to perform three separate statistical tests [threshold number of misclassifications (TNoM), Student's $t$ test, and the mutual information score (Info) (44)] to select genes with significant changes in expression ($P < 0.05$ for all three tests). We then used standard hierarchical clustering methods (PCluster in SCOREGENES) to compare gene expression patterns. After selecting clusters that exhibited the greatest differences in expression between unmated and mated females, we used a hypergeometric model in GENEXPRESS to compute the probability of finding enrichment of specific GO annotation categories in a specified cluster.

**Proteomic Assays.** We dissected lower reproductive tracts of unmated and mated females at 3 h postmating as described above and stored them at −20°C for pooling. Samples were precipitated in cold acetone and centrifuged at 8,500 × *g* for 10 min. Protein pellets were solubilized in isoelectric running buffer. 2D-PAGE was performed by using a protean two-dimensional electrophoresis system (Bio-Rad). We loaded 200 μg of the protein sample onto individual pH 3–10, nonlinear, 11-cm immobilized pH gradient strips (Bio-Rad). The second dimension of the electrophoresis was carried out by using precast gradient gels of 4–20% polyacrylamide (Criterion; Bio-Rad). We visualized gels with SeeBand staining (GeBa); images were taken with a FluorS imager (Bio-Rad).

We analyzed images with PDQUEST 7.0.1 (Bio-Rad). Spots were automatically detected in each experimental gel and were matched to their corresponding spots in a digitized reference gel. We normalized protein spot intensity levels between gels by

dividing the spot intensity level by the sum of the intensities of all of the spots in the gel. Spots were cut and in-gel reduced with 10 mM DTT, incubated at 60°C for 30 min, and then digested with trypsin overnight at 37°C. We resolved tryptic peptides by reverse-phase chromatography (Applied Biosystems). Peptides were eluted with linear gradients of 5 to 95% acetonitrile for 80 min. Mass spectrometry was performed with ion-trap mass spectrometers (LCQ and LCQ-DecaXP; Thermo, San Jose, CA). Data were analyzed by using PEP-MINER (45) as described below.

For LC-MS/MS, total protein extract prepared as described above for 2D-PAGE analysis was denatured and alkylated before digestion overnight at 37°C by using modified trypsin (Promega) at a 1:100 enzyme-to-substrate ratio. Trypsinized total protein extracts were desalted, eluted with 90% acetonitrile, dried, and dissolved in 0.1% formic acid. Aliquots of the peptides were analyzed by multidimensional protein identification technology (MudPIT). Eluted fractions were dried again by vacuum centrifugation and dissolved in 0.1% acetic acid for one-dimensional $\mu$LC-ESI-MS/MS (capillary chromatography electrospray ionization tandem mass spectrometry). The mass spectrometry data were clustered and analyzed by using PEP-MINER and SEQUEST, and searched against the *Drosophila* Protein and the National Center for Biotechnology Information (NCBI) databases. Results from SEQUEST were considered significant when $\Delta$Cn was equal to 0.1 or greater. Accuracy of identification of individual peptides was assessed visually. Protein analysis and identification were carried out at the Smoler Proteomics Center at the Technion (Israel Institute of Technology, Haifa, Israel).

**Comparison of Proteomic and Genomic Data Sets.** To match the genes represented on the genomic microarrays with corresponding gene products identified in the proteomic experiments, we compared the proteomic (2D-PAGE and LC-MS/MS) and genomic [genes that were present at least twice (MAS 5.0, Affymetrix) in at least one treatment (unmated and 0, 3, 6, or 24 h since mating)] data sets. We next categorized the 84 differentially expressed proteins combined from both 2D-PAGE and LC-MS/MS analyses as follows: proteins with transcript present in unmated and all mated female time points were scored as positive, proteins that did not have a corresponding transcript in unmated or any mated female time points were scored as negative, and proteins with transcript in a subset of unmated and mated female time points were scored as intermediate. We then determined: (*i*) whether or not we had observed transcript for the proteins identified in the lower reproductive tract and (*ii*) how many of the differentially expressed proteins had transcripts present that were also differentially expressed.

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