Current Biology, Vol. 14, 1509–1514, August 24, 2004, ©2004 Elsevier Ltd. All rights reserved. DOI 10.1016/j.cub.2004.08.028

Genes Regulated by Mating, Sperm, or Seminal Proteins in Mated Female *Drosophila melanogaster*

Lisa A. McGraw,[1] Greg Gibson,[2] Andrew G. Clark,[1] and Mariana F. Wolfner[1,*]

[1]Molecular Biology and Genetics
Cornell University
Ithaca, New York 14853

[2]Department of Genetics
North Carolina State University
Raleigh, North Carolina 27695

Summary

In *Drosophila melanogaster*, sperm and accessory gland proteins ("Acps," a major component of seminal fluid) transferred by males during mating trigger many physiological and behavioral changes in females (reviewed in [1–5]). Determining the genetic changes triggered in females by male-derived molecules and cells is a crucial first step in understanding female responses to mating and the female's role in postcopulatory processes such as sperm competition, cryptic female choice, and sexually antagonistic coevolution. We used oligonucleotide microarrays to compare gene expression in *D. melanogaster* females that were either virgin, mated to normal males, mated to males lacking sperm, or mated to males lacking both sperm and Acps. Expression of up to 1783 genes changed as a result of mating, most less than 2-fold. Of these, 549 genes were regulated by the receipt of sperm and 160 as a result of Acps that females received from their mates. The remaining genes whose expression levels changed were modulated by nonsperm/non-Acp aspects of mating. The mating-dependent genes that we have identified contribute to many biological processes including metabolism, immune defense, and protein modification.

Results and Discussion

We used Affymetrix GeneChips to identify genes whose expression in *D. melanogaster* females is modulated 1–3 hr postmating—when the first physiological effects of Acps and sperm on females are detected [6–8]. Hybridization data were analyzed using a probe-based linear model [9] with a test-wise significance threshold of $p < 0.05$ for each treatment contrast. Expression of ~13% of the transcriptome was found to be modulated by mating. As described in the supporting experimental procedures, up to one quarter of these differences may be false positives, as assessed by q values [10]. Verification of a small number of changes was performed using quantitative reverse-transcription PCR (see Supplemental Data). 51% of genes can be assigned known or predicted functions based on sequence comparisons or database information (Table 1).

Given the dramatic changes in a female's physiology and behavior after mating, it was possible that large gene expression changes might be triggered by mating. Alternatively, unmated, but sexually mature, females might already be "poised" to respond to mating due to prior expression of genes whose products are activated by, or needed to respond to, sperm or Acps. In this case, we would expect gene expression changes to be small. Only 46 of the 1783 genes exhibited 2-fold or greater changes in RNA levels after mating (Table 2), suggesting that females are poised to respond quickly to mating without needing to modulate gene activity. A similar model is favored by observations on peptidergic vesicle dynamics following mating: release of contents of pre-existing peptidergic vesicles is regulated by Acps, sperm, or mating [11]. Our results suggest that genes whose products are needed for early postmating responses may have been expressed during sexual maturation; mating may simply activate their products to allow an immediate and rapid response. This would ensure that processes such as moving sperm into storage can begin rapidly. Examination of females at later times postmating might show greater changes in expression levels. The small magnitude of mating-induced gene expression changes could also reflect our use of whole flies. Although this eliminated tissue-biased observations, a large gene expression change in one tissue could have been diluted by constant levels in other tissues. Subsequent experiments to determine tissue specificity of expression of mating-regulated genes can address this.

Specific Components of Mating Regulate Different Subsets of Genes in Females

To determine the separate effects of male contributions to mating (sperm, Acps, and other components of mating) on female gene expression, we mated females to spermless males (sons of *tudor* females) or to males that lack both sperm and Acps (DTA-E). Previous work using these fly strains showed that sperm and Acps modulate changes in females, including reducing female receptivity to remating, stimulating oogenesis and egg laying, facilitating sperm storage, regulating sperm competition, and decreasing the female's lifespan (see [1–5] and references therein).

To determine if a gene's expression level is regulated by sperm, Acps, or other aspects of mating, we examined pair-wise differences in expression values according to the schema in Figure 1. Of the 1783 genes that differed in expression levels between virgin and mated females, 160 are regulated by Acps and 549 by sperm. Nonsperm/non-Acp cues regulate the remaining 1074 genes. Genes regulated by nonsperm/non-Acp cues may be regulated by the physical act of mating [12], by energy costs incurred as a result of courtship and mating, by chemical cues from males, or by other

*Correspondence: mfw5@cornell.edu

Table 1. Functional Classification of Genes Based on Gene Ontology

Gene Ontology	Nonsperm/ Non-Acp Regulated	Acp Regulated	Sperm Regulated
Actin binding	0.3% (3)	-	1.1% (6)
Calcium binding	0.2% (2)	0.6% (1)	0.7% (4)
Cell adhesion	0.3% (3)	-	0.4% (2)
Cell cycle regulator	-	-	0.4% (2)
Chaperones	0.3% (3)	-	1.3% (7)
Cytochrome P450	1.4% (15)	1.3% (2)	2.0% (11)
Defense/Immunity	0.5% (5)	6.3% (10)	2.6% (14)
Electron transfer	-	-	0.7% (4)
Enzymes	13.0% (140)	13.8% (22)	22.0% (121)
Ion channel	0.8% (9)	-	-
Ligand binding or carrier	1.8% (19)	3.8% (6)	2.9% (16)
Motor protein	0.6% (6)	-	1.3% (7)
Nucleic acid binding	2.9% (31)	1.9% (3)	1.8% (10)
Proteases	4.7% (51)	3.1% (5)	5.8% (32)
Protease inhibitors	0.2% (2)	-	-
Protein kinase	1.3% (14)	1.9% (3)	2.0% (11)
Receptor	3.0% (32)	4.4% (7)	1.3% (7)
Signal transduction	0.8% (8)	-	-
Structural protein	1.8% (19)	0.6% (1)	2.4% (13)
Transcription factors	3.7% (40)	1.9% (3)	2.2% (12)
Transporters	3.0% (32)	4.3% (7)	5.3% (29)
Other	4.6% (49)	5.6% (9)	7.1% (39)
Unknown	55.0% (591)	50.6% (81)	36.8% (202)
Total number of genes	1074	160	549

Percentages represent the percent of genes from each category of genes (those modulated by nonsperm/non-Acp cues, those modulated by Acps, or those modulated by sperm) that fall into each functional group. The number of genes in each of these classifications are in parentheses.

ejaculate components (i.e., products of the ejaculatory duct or bulb).

Below, we focus on several classes of genes chosen based on significant overrepresentation in the data set (assessed using EASE [13]) and a few whose predicted functions are congruent with known physiological changes in mated female flies.

Metabolism and Enzymes

RNAs encoding metabolism genes are overrepresented in our data set (EASE score, $p < 0.0001$) largely in response to sperm or to nonsperm/non-Acp cues. Sperm downregulate the expression of 11 putative NADH dehydrogenase genes (CG3192, CG12203, CG3214, CG9306, CG2286, CG7712, CG6463, CG6343, CG15434, CG5703, and CG12079), and sperm and nonsperm/non-Acp cues downregulate several antioxidant genes including cytochrome c oxidases (*CoVa*, CG10664, CG11015, CG17280 by sperm), oxidoreductases (CG3301, CG3495, CG3609, CG9331, CG12171, and CG12224 by nonsperm/non-Acp cues), and glutathione transferases (*Gst3-1*, *Mgstl*, CG17527, and CG1681 by sperm and CG17525 and CG6776 by nonsperm/non-Acp cues). Occasionally, a single member of these families is upregulated (for example, 1 NADH dehydrogenase, CG6914, by nonsperm/non-Acp cues; 1 glutathione transferase, CG17639, by Acps). In addition, the expression levels of four lysozyme genes (*LysB*, *LysC*, *LysD*, and *LysE*) are decreased by the presence of sperm.

Postmating modulation of the female's metabolism likely contributes to several phenomena. First, of the ~4000 sperm that females receive from their mates, ~1000 are maintained in storage organs to await opportunities to fertilize eggs [14, 15]. Females must provide an environment supportive of sperm viability and motility and must counter oxidative effects of sperm catabolism [16, 17]. Second, increased egg production in mated females might require that females allocate resources away from somatic maintenance and invest resources in reproductive processes [18]. The overall downregulation of metabolic genes by mating may reflect a trade-off between reproductive and nonreproductive processes.

Cytochrome P450s

22 cytochrome P450 genes are downregulated by mating in females, more than expected by chance (EASE score, $p = 0.0001$). Nine are downregulated by receipt of sperm (*Cyp4d1*, *Cyp6a13*, *Cyp6a17*, *Cyp6a21*, *Cyp6g1*, *Cyp9f2*, *Cyp9f3*, *Cyp12a4*, and *Cyp12a5*), while 13 are downregulated by nonsperm/non-Acp components of mating (*Cyp4ac1*, *Cyp4ac2*, *Cyp4e2*, *Cyp4p1*, *Cyp6a2*, *Cyp6a23*, *Cyp6d5*, *Cyp6t1*, *Cyp6w1*, *Cyp9b2*, *Cyp28d1*, *Cyp309a1*, and *dib*). Six cytochrome P450 genes are upregulated in mated female flies (*Cyp9f3*, *Cyp307a1*, and *Cyp315a1* by sperm, *Cyp4p3* and *Cyp313a4* by Acps, and *Cyp6a21* by nonsperm/non-Acp cues).

Insect cytochrome P450s comprise a diverse class of enzymes involved in detoxification and in biosynthesis of ecdysteroids and juvenile hormones [19, 20]. Downregulation of cytochrome P450 genes in mated females could reflect several processes. First, it might be important not to "detoxify" metabolites and molecules received in seminal fluid from males. Second, although juvenile hormone synthesis increases after mating due

Table 2. Genes that Exhibit 2-Fold or Greater Changes in Expression in at Least One Comparison as a Result of Acps, Sperm, and Nonsperm/Non-Acp Cues

Gene Identity	Expression Difference	p Value	Expression Difference	p Value	Gene Ontology
Genes Regulated by Acps	Mated-Virgin	Mated vs. Virgin	Mated to DTA-Mated to *tudor*	Mated to DTA vs. Mated to *tudor*	
AttB	0.9040	0.0001	−1.6319	<0.0001	Defense/Immunity
AttA	1.0757	<0.0001	−1.5530	<0.0001	Defense/Immunity
CecA2	0.6650	0.0010	−1.0191	<0.0001	Defense/Immunity
CecA1	0.5860	0.0158	−1.0099	0.0001	Defense/Immunity
IM1	1.0032	0.0004	−0.8250	0.0015	Defense/Immunity
Genes Regulated by Sperm	Mated-Virgin	Mated vs. Virgin	Mated to Wild-Type-Mated to *tudor*	Mated to Wild-Type vs. Mated to *tudor*	
Amy-d	−1.1312	<0.0001	−1.0134	<0.0001	Calcium binding
CG12408	−1.4072	0.0165	−1.8888	0.0014	Calcium binding
Dro	1.0782	0.0001	1.1580	<0.0001	Defense/Immunity
Mtk	0.9380	0.0018	0.9402	0.0017	Defense/Immunity
LysC	−0.9422	0.0043	−1.0018	0.0024	Enzyme
LvpH	−2.2048	<0.0001	−0.9990	0.0149	Enzyme
Uro	1.0471	<0.0001	0.2129	0.0444	Enzyme
CG6295	−0.9881	0.0054	−1.2781	0.0003	Enzyme
CG8690	−1.2318	<0.0001	−0.4798	0.0104	Enzyme
CG11909	−1.7528	<0.0001	−0.5873	0.0162	Enzyme
CG15434	−1.1467	0.0065	−1.4099	0.0009	Enzyme
Act88F	−1.6771	0.0023	−2.9331	<0.0001	Motor protein
Mlc1	−0.4515	0.0226	−1.3157	<0.0001	Motor protein
Mlc2	−0.5934	0.0151	−1.3769	<0.0001	Motor protein
alphaTry	−1.2501	<0.0001	−0.9738	0.0004	Protease
betaTry	−1.2530	<0.0001	−1.0218	0.0005	Protease
epsilonTry	−0.9533	0.0019	−0.9651	0.0017	Protease
CG6298	−1.3108	<0.0001	−1.3314	<0.0001	Protease
CG12374	−1.4521	0.0002	−1.5671	0.0001	Protease
CG13095	−1.5437	0.0001	−0.9467	0.0125	Protease
fln	−1.3550	0.0260	−2.3716	0.0001	Structural protein
CG6484	−1.3815	<0.0001	−0.7747	0.0035	Transporter
CG9090	−0.8550	0.0181	−1.4669	0.0001	Transporter
Smp-30	−1.0054	<0.0001	−0.3085	0.0303	Other
CG3348	1.2503	<0.0001	0.9530	<0.0001	Unknown
CG4363	−1.9590	<0.0001	−0.8353	0.0090	Unknown
CG4377	−1.1203	<0.0001	−0.3595	0.0172	Unknown
CG7953	−1.4839	<0.0001	−0.5179	0.0450	Unknown
CG7916	−1.2765	<0.0001	−0.6127	0.0218	Unknown
CG8689	−1.6601	<0.0001	−0.9361	0.0002	Unknown
CG13947	−0.6140	0.0220	−1.1656	<0.0001	Unknown
CG13323	−1.1533	0.0001	−0.7247	0.0142	Unknown
CG13323	−1.0110	0.0002	−0.5334	0.0445	Unknown
CG14125	−1.2052	0.0001	−0.9966	0.0013	Unknown
CG17820	1.0834	<0.0001	0.8840	0.0001	Unknown
Genes Regulated by Nonsperm/Non-Acp Components of Mating	Mated-Virgin	Mated vs. Virgin			
Cyp6d5	−1.0124	<0.0001			Cytochrome P450
CG11669	−1.4366	<0.0001			Enzyme
CG7542	−1.3575	<0.0001			Protease
CG18125	1.2069	<0.0001			Protease
CG10299	−1.0431	<0.0001			Unknown
CG15043	−1.0036	0.0005			Unknown

Expression difference is denoted in $-\log_2$ (e.g., a value of 1.0 represents a 2-fold change in gene expression).

to seminal proteins [21], this is likely a transient effect; modulation of cytochrome P450s could be necessary to regulate subsequent hormone levels. Finally, down-regulation of cytochrome P450 genes may be part of a trade-off whereby females allocate resources away from detoxification and toward reproduction.

Immune/Defense Response

Mating regulates RNA levels of 19 immunity-related genes—more than expected by chance (EASE score, $p < 0.0001$). Most of these genes encode known or putative antimicrobial peptides characteristic of the humoral immune response in *Drosophila*. Though non-

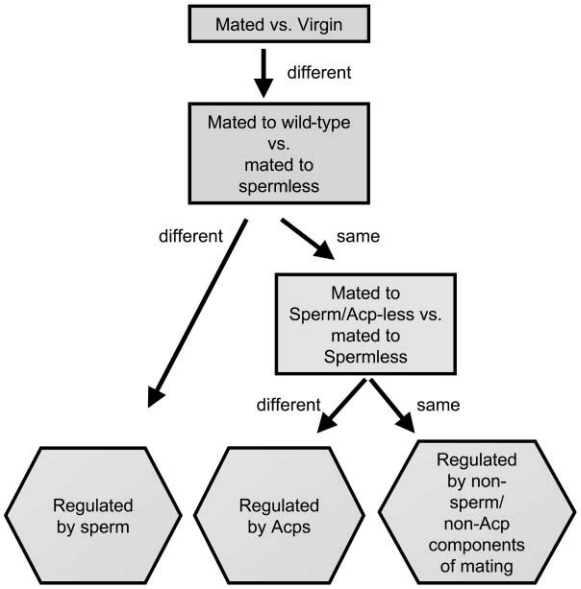

Figure 1. Identifying Mating-Responsive Genes Regulated by Sperm, Acps, or Nonsperm/Non-Acp Cues

To determine if a gene's expression level is regulated by sperm, Acps, or nonsperm/non-Acp aspects of mating, we examined pairwise differences in expression values according to the schema shown. Among the 1783 genes whose expression levels differed between mated and virgin females, we then identified those genes exhibiting significant differences in expression between females mated to wild-type males versus females mated to spermless males. Since the only difference between these crosses is the presence or absence of sperm, this comparison revealed the genes regulated in females by receipt or presence of sperm. The genes whose expression levels differed between mated and virgin females but were not regulated by sperm were then subdivided into those whose expression levels differed significantly between females mated to males lacking sperm and Acps compared to females mated to spermless males. Since the only difference between these two crosses was the absence of Acps in the former, genes that differed in expression in this comparison are regulated by Acps. The remaining genes whose expression levels are modulated by mating, but not by either sperm or Acps, must be regulated by some other component of mating.

sperm/non-Acp cues decrease expression of two putative antimicrobial genes (CG12780 and CG16756), sperm upregulate expression of eight antimicrobial genes (*AttC*, *Dpt*, *Dro*, *Mtk*, *PGRP-SA*, *PGRP-SB1*, *18w*, and *TotM*) and Acps upregulate the expression of nine antimicrobial genes (CG13422, *AttA*, *AttB*, *AttD*, *CecA1*, *CecA2*, *CecB*, *IM1*, and *IM2*).

Correlations between reproduction and immune response have been well documented in *Drosophila* and other species but have primarily been explored in terms of a cost to males [22, 23]. Although the female reproductive tract of *Drosophila* constitutively expresses several antimicrobial peptides [24, 25], our data indicate that mating induces an immune response in females. Therefore, sperm and/or Acps may be perceived as foreign molecules/cells or may be accompanied by microorganisms that elicit a female immune response.

Proteolysis Regulators

Postmating expression levels of 88 protease genes change; they are overrepresented in our data set (EASE score, $p < 0.0001$). Of these, 28 encode predicted serine-type peptidases, 16 of which are downregulated after mating. Receipt of sperm induces expression of two of these genes (*ndl* and CG6639) and downregulates 12 others (*Ser6*, CG5390, CG6467, CG6483, CG10472, CG10477, CG11911, CG11912, CG13095, CG16749, CG16997, and CG17571). Sperm also downregulate five trypsin genes (*alphaTry*, *betaTry*, *epsilonTry*, *kappaTry*, and *thetaTry*) and two chymotrypsin genes (CG6298 and CG8871). Nonsperm/non-Acp cues upregulate RNA levels of eight serine-type peptidase genes (*gd*, CG1299, CG8170, CG13318, CG14760, CG14990, and CG18124) and downregulate four genes (*Ser99Dc*, CG8952, CG9673, and CG16996). Nonsperm/non-Acp cues also downregulate three trypsin genes (*iotaTry*, *lambdaTry*, and *Try29F*, but not *zetaTry*) and two chymotrypsin genes (CG7542 and CG8871, but not CG18030). Acps do not appear to regulate trypsin or chymotrypsin genes, but weakly upregulate expression of one serine-type endopeptidase (CG5909). Two protease inhibitors are downregulated by sperm (CG12955) and by nonsperm/non-Acp cues (CG8050).

Modulating expression of proteolysis regulators in mated females could impact several physiological processes. First, proteolytic cascades regulate the immune response; this modulation could synergize with the mating induction of antimicrobial peptide genes noted earlier. Second, induced proteases could protect females from harmful proteins introduced during mating. Third, at least two Acps are proteolytically processed in the female reproductive tract [26, 27]. This regulation could release active subregions of these proteins or control or terminate activities of these Acps. Modulation of proteases by females is consistent with data showing that the processing of Acp26Aa involves both male and female contributions [28]. Fourth, proteases may expose protein regions in the female reproductive tract or assist in the formation or degradation of the mating plug. Finally, protease inhibitors may protect sperm from degradation or expose sperm surface proteins needed for storage or fertilization.

Other Classes of Mating-Regulated Genes

Expression of ten genes encoding predicted pheromone or odorant binding proteins changes as a result of sperm (*Obp56a*, *Os-c*, and *Pbprp3*) or nonsperm/non-Acp cues (*Obp50e*, *Obp56g*, *Opb59c*, *Opb83c*, *Obp99c*, *Pbprp2*, and *Pbprp5*). Modulation of these proteins could contribute to postmating changes in the female's attractiveness or receptiveness to courting males [29, 30] and potentially to the selection of oviposition sites [31].

Receipt of sperm decreases RNA levels for five muscle motor genes (*Act88F*, *Mhc*, *Mlc1*, *Mlc2*, and *Tm2*). This may reflect modulation of muscle density to effect contractility changes that may occur in the reproductive tract to move eggs or to retain sperm in storage.

Nonsperm/non-Acp cues upregulate the expression of 40 transcription factors (see Supplemental Table S3). These proteins could trigger gene cascades that cause subsequent steps in the female's physiological switch from virgin to mated. Although some of these transcription factors are expressed during early embryogenesis,

since we sampled females 1–3 hr after a first mating, it is unlikely that any embryos were present in our sample. Thus, certain developmentally regulated transcription factors could also regulate nonembryonic functions such as aspects of the mating response.

Sperm, Acps, or Nonsperm/Non-Acp Components of Mating Regulate Unique Sets of Genes

Although there is overlap in the classes of female genes that are modulated by different male-derived components of mating, we find that sperm, Acps, and nonsperm/non-Acp cues have distinct characteristics with respect to the genes they modulate. For instance, although immune peptide genes are modulated by all components of mating, Acps strongly upregulate (2-fold or greater expression change) five of these ten genes. The magnitude of expression change of immune peptide genes modulated by Acps is striking compared to those modulated by sperm or nonsperm/non-Acp cues. Sperm downregulate 11 NADH dehydrogenase genes—a gene class that is not among the genes regulated by Acps and is represented a single time (and, upregulated) among genes regulated by nonsperm/non-Acp cues. Sperm also uniquely downregulate hydrogen-transporting ATPases. Finally, nonsperm/non-Acp cues upregulate 40 transcription factors, but only a few are modulated by sperm (12 genes) or Acps (3 genes).

Overlap in Male and Female Genic Contributions to Mating Processes

Several classes of genes regulated in females by mating overlap with known or predicted classes of proteins provided to females in seminal fluid. Examples include antibacterial peptides, proteases, protease inhibitors, and lipases [32]. Surprisingly, we also detected two Acp genes that are downregulated in females by mating: a putative lipase (CG8093) and a putative endopeptidase (CG4847). Other evidence (K. Ravi Ram, M. Ramakrishnan, L.A.M., and M.F.W., unpublished data) corroborates the finding that these two Acps are expressed at low levels in females. Two putative glucose dehydrogenase genes are upregulated in mated females: CG12398 (by sperm) and CG9517 (by nonsperm/non-Acp cues). Glucose dehydrogenase (*Gld*) is expressed in the female sperm storage organs and vaginal plate and also in the male's ejaculatory duct, from which it is also transferred to females [33]; both appear to contribute to sperm storage in females.

Conclusions

Our genome-wide analysis of the early postmating response in female *D. melanogaster* has identified gene expression changes that occur, separately, in response to sperm, Acps, and nonsperm/non-Acp cues. Our data provide a framework for understanding female roles in mating and in postcopulatory sexual selection. Although many of the expression changes that occur in females likely regulate fundamental aspects of reproduction, other genes may be modulated in the interest of the male or may be regulated to counteract manipulation by the male.

Supplemental Data

Supplemental data including experimental procedures and several tables can be found at http://www.current-biology.com/cgi/content/full/14/16/1509/DC1.

Acknowledgments

We thank the University of Kentucky Microarray Core Facility for processing of GeneChips; T. Chu, J. Ewer, J. Scott, P. Wittkopp, and R. Wolfinger for valuable discussions; E. Adams, K. Sackton, A. Fiumera, V. Horner, J. Mueller, A. Saunders, T. Schlenke, A. Wong, and anonymous reviewers for helpful comments on the manuscript; and funding from NIH grant HD-38921 to M.F.W, NSF grant DEB-0108965 and NIH grant AI46402 to A.G.C., and a traineeship on NIH Training Grant GM07616 to L.A.M.

Received: May 17, 2004
Revised: July 1, 2004
Accepted: July 1, 2004
Published: August 24, 2004

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Note Added in Proof

While this paper was in proof, a paper by Lawniczak and Begun appeared online (Lawniczak, M.K.N., and Begun, D.J. (2004). A genome-wide analysis of courting and mating responses in *Drosophila melanogaster* females. Genome *47*, 1–11). These authors also report mating-induced genes with *D. melanogaster* females; 55.3% of the 38 genes they describe overlap with genes that we report here.