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The Influence of Photoperiod and Mating on Profiles of Seminal Fluid Peptides from Male Accessory Glands of *Helicoverpa armigera*

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ABSTRACT

After mating many female insects undergo physiological and behavioral changes including inhibition of receptivity to further matings and induction of oviposition. These changes are modulated by the transfer of proteins from male seminal fluids during mating. Here, we identified a number of proteins in the seminal fluid secretion of the moth species, *Helicoverpa armigera* that undergo changes in accordance to photoperiod and are reduced or depleted after mating, thereby indicating a probable functional significance during mating. Changes in seminal fluid proteins were studied using LC-MS/MS and 2D SDS PAGE to identify proteins that are both up-regulated during the scotophase and depleted after mating. A total of 98 proteins were identified using LC-MS/MS, out of which the levels of 52 were up-regulated during the scotophase**.** We identified many functional similarities to seminal fluids from other insects. Thus, although seminal fluid proteins are amongst the most rapidly evolving proteins, the classes they represent are relatively conserved. The proteins identified were classified into 7 different functional groups: signal transduction; immune function; lipid transporting; gene function; cytoskeletal proteins; apoptosis; metabolism; the remainder of unknown function**.** More than half of the identified proteins were up-regulated during the scotophase with most at a level of up to 5 fold but 7 of the proteins were up-regulated to levels of more than 20 fold**.** Using comparative 2D electrophoresis MAG content was further studied to identify extracted proteins that are both up-regulated during the scotophase and depleted after mating and thus may be transferred to the female during copulation. Most of the identified *H. armigera* proteins bore high homologies to proteins in both the *Bombyx mori* and the *Drosophila*

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melanogaster databases. The possible individual roles of these proteins in the mating process are discussed.

KEYWORDS**:** male accessory glands;- mating behavior;- photoperiod; 2D-SDS-PAGE; LC-MS/MS

INTRODUCTION

Reproductive success in several moth species depends on attraction through species-specific sex-pheromones that are synchronously produced and released, usually by the female, for mate finding. This synchronization is regulated in many Lepidopteran species by Pheromone Biosynthesis Activating Neuropeptide (PBAN) (Rafaeli, 2009) through its direct interaction with the PBAN-receptor (PBAN-R) present in the pheromone gland, which is situated between the ultimate and penultimate abdominal segments (Jurenka and Rafaeli, 2011). Sexually receptive adult female moths produce and emit the pheromone blend usually during the scotophase by typical calling behavior involving the extrusion of the sex-pheromone gland. Males, perceiving the sex-pheromone, orient to receptive females and mating occurs. In the moth, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) we previously showed that mating induces depletion of PBAN levels in the hemolymph and a reduction in the pheromone levels produced by females (Nagalakshmi *et al*., 2007).

After mating many insects undergo physiological and behavioral changes (Avila *et al*., 2011; Chen, 1984; Gillott, 2003; Kubli, 2003) including inhibition of receptivity (permanently or transiently), induction of oviposition, induction of immune peptide expression, changes in female flight and feeding behavior, modulation of sperm storage parameters and reduction in the female's lifespan. Many of these changes are modulated by the transfer of seminal fluid proteins and other components during mating that are produced in the male reproductive tract. The seminal fluid proteins identified to date represent various protein classes, including lectins, pro-hormones, proteases/protease inhibitors, protective proteins such as antioxidants, and peptides (Avila *et al*., 2011; Chapman and Davies, 2004; Gillott, 2003;).

An extensive study on the seminal fluid proteins in the fruit fly *Drosophila melanogaster* (Meigen) (Diptera: Drosophilidae) has thus far identified 146 of these proteins, postulated to be present in the *D. melanogaster* genome. Many of these genes encode proteins containing signal peptides for secretion and show male-specific expression that is highly enriched in the male accessory glands (MAGs) (Avila *et al*., 2011; Mueller *et al*., 2005; Swanson *et al*., 2001). They include some of the most rapidly evolving genes in the *Drosophila* genome (Swanson *et al*., 2001), therefore numerous attempts to identify their orthologs in other insects have failed (Andres *et al*., 2006; Collins *et al*., 2006; Davies and Chapman, 2006). However, these protein classes are seen in the ejaculates of several insect species, suggesting that, even though the primary sequence of some proteins evolve rapidly, the protein classes represented in the seminal fluid are constrained (Mueller *et al*., 2005). Of all *Drosophila* MAG proteins there are three

peptides that are considered to be very important in eliciting increased fecundity and in causing non-receptivity after mating: sex peptide (*Drm*SP-Acp70A), ovulin (Acp26Aa) and ductus ejaculatorius peptide (DUP99B) (Chen *et al*., 1988; Herndon and Wolfner, 1995; Saudean *et al*., 2002). In *Drosophila*, injection of purified *Drm*SP and its ectopic expression in virgin females induced stimulation of egg production and a decrease in receptivity for 1-2 days (Aigaki *et al*., 1991; Chen *et al*., 1988; Nakayama *et al*., 1997).

Correspondingly, in several female moths, mating results in a loss of sexual receptivity which is manifested through the depletion of sex-pheromone production (pheromonostasis) and absence of calling behavior (Kingan, 1995; Rafaeli, 2002; 2005; Raina, 1993; Ramaswamy *et al*., 1994). The synthetic form of *Drm*SP and its peptide analogs are cross reactive in *H. armigera* virgin female moths (Fan *et al*., 1999; 2000) and inhibit pheromone production. In addition, injection of an endogenous, partially purified *Drm*SP-like peptide, identified from *H. armigera* MAGs, caused sex-pheromone suppression in virgin females (Eliyahu *et al*., 2003; Fan *et al*., 1999; 2000; Nagalakshmi *et al*., 2004). Moreover, immunoassays on purified MAG extracts showed that the total immunoreactivity and the number of *Drm*SP-immunoreactive peaks increase in the MAGs during the active mating hours of the scotophase and decrease after mating (Nagalakshmi *et al*., 2007). We also recently showed that *Drm*SP has a stimulatory effect on oviposition and a significant reduction in the gene expression levels of the PBAN-R. In addition, we identified the *Hea*SP-receptor (*Hea*SP-R) and demonstrated a significant up-regulation in gene expression levels of this receptor in brains and pheromone glands of mated females (Hanin *et al*., 2011). Silencing of this *Hea*SP-R through RNA-interference prevented *Drm*SP-induced suppression of pheromone production and calling behavior (Hanin *et al*., 2012). Moreover, mated, silenced females failed to increase their oviposition rates as is normally observed in mated females, and their behavior did not differ from that of virgin females (Hanin *et al*., 2012). However, sex pheromone production by mated, silenced females remained suppressed as in normal mated females, thereby indicating the probable involvement of additional factors in the suppression of sex pheromone production after mating (Hanin *et al*., 2012). These findings suggest that various seminal fluid proteins have a strong impact on the sexual behavior of *H. armigera* females.

In the present study we start to understand the dynamic changes in protein profiles that occur during the photoperiod and after mating by identifying several *H. armigera* MAG proteins using two-dimensional polyacrylamide gel electrophoresis and LC-MS/MS.

MATERIALS AND METHODS

2.1. Insect culture

H. armigera larvae were raised on an artificial diet (Heliothis Premix, Stonefly Industries, Inc, Bryan, TX, USA) in transparent culture cells (J-2 cavities, Nu-Trend Container, Jacksonville, FL, USA) at a constant temperature of 26 ± 1 °C and 14:10

h (light: dark) photoperiod as reported previously (Rafaeli *et al*. 2003). Pupae were sexed and adult males and females were allowed to emerge in separate cages, containing 10% sugar water until tissue dissection.

2.2. LC-MS/MS identification of MAG leached proteins

Male accessory glands including the duplex and simplex were dissected from 3-5 day-old naïve males during the 6^{th}-8^{th} hours of the scotophase (at peak mating activity) and during the 7^{th}-9^{th} hours of the second photophase (when no mating activity occurs). These were immediately incubated in a physiological medium pH 6.6 containing: 5mM Pipes buffer, 18mM magnesium chloride, 21mM potassium chloride, 12mM sodium chloride, 3mM calcium chloride, 85mM glucose, 43mM trehalose and 0.001M phenol red (Jurenka *et al*., 1991) (1 MAG/ 50 ml medium) on ice for one hour. Subsequently the MAGs were discarded and the medium was frozen until analysis. Samples of proteins, released into the physiological medium from a pool of two MAG equivalents were identified and quantified by dimethylation and LC-MS/MS at the Smoler Proteomics Center (Technion Israel Institute of Technology, Department of Biology). After trypsin digestion and desalting the resulting peptides were labeled by dimethylation in the presence of 100mM $NaCBH_3$ (1M), by adding Light Formaldehyde (35% w/w, 12.3M) to the control samples (photophase), and Heavy Formaldehyde (20% w/w, 6.5M) to the experimental samples (scotophase). After 5 hours of incubation the samples were neutralized and equal amounts of the light and heavy peptides were mixed, purified on C18 tips (Ultra-Micro PrepTip™ , 1-10mL, C18-Vydac, Harvard Apparatus, USA) and re-suspended in 0.1% formic acid. The peptides were resolved by reverse-phase chromatography on 0.075 X 200-mm fused silica capillaries (J & W) packed with Reprosil reversed phase material (Dr Maisch GmbH, Germany). The peptides were eluted using linear 90 min. gradients of 5 to 45% and 15 min. at 95% acetonitrile containing 0.1% formic acid in water at flow rates of 0.25 ml/min. Mass spectrometry was performed by an ion-trap mass spectrometer (Orbitrap, Thermo) in a positive mode using repetitively full MS scan followed by collision induced dissociation (CID) of the 7 most dominant ion selected from the first MS scan. The mass spectrometry data between photophase and scotophase duplicate samples relative level of up-regulation during the scotophase was calculated (photophase/scotophase ratio). The data was analyzed using the Sequest 3.31 software (Eng, *et. al*., 1994; Finnigan, San Jose) searching against the Insecta part of the NCBI-NR database. Peptide sequences identified were validated using NCBI protein databases.

2.3. Mating Experiments

Mating experiments were performed using two-day-old virgin females and three-day-old naïve (unmated) males during the scotophase. Insects were allowed to mate in mating cages at 5-6 h after the onset of the scotophase. Mating pairs were carefully removed from the mating cage and kept in Petri dishes for observation. The pairs were

checked every 10 min. Only pairs that remained in copula for at least 90 min, were taken into consideration. On completion of mating, the mated males were examined for a complete depletion of duplex contents and the mated females were dissected for the presence of an intact spermatophore in their bursa-copulatrix.

2.4. Two-Dimensional Polyacrylamide Gel Electrophoresis (2D-PAGE)

Male accessory glands were dissected from naïve males during the photophase and scotophase and from mated males during the scotophase. The tissues were collected in Tris buffer (5mM Tris, pH 7.5) containing a mixture of protease inhibitors (Complete EDTA-Free protease inhibitor from Roche Diagnostics GmbH, Mannheim, Germany). They were subsequently frozen using liquid nitrogen and stored at -70°C until extracted. The glands were centrifuged at 10,000g for 20 min at 4^0C and the supernatant was used for electrophoresis. MAG extracts were examined by 2D-PAGE on 12% polyacrylamide gels and Tris-Tricine 2D-PAGE on 16% gels (in triplicates).

2.4.1. First Dimension

The protein samples were added to rehydration buffer containing 9M urea, 3% CHAPS, 0.002% Bromophenol blue, 2% (v/v) linear 3-10 IPG buffer, 50mM DTT and 0.5% Triton-X-100. Two hundred to six hundred micrograms of protein were loaded on an IPG strip (13cm, 3-10 pH, Linear, GE Healthcare, Uppsala, Sweden) via passive rehydration using immobiline dry strip re-swelling tray for 16 h at 30^0C. First dimension IEF was performed in the Multiphor II System (Amersham Pharmacia-Biotech, Uppsala, Sweden). Focusing was performed at 300V for 15 min, 500V for 15 min, 1000V for 15 min, 1500V for 15 min, 2000V for 15 min, 2500V for 15 min, 3000V for 15 min and 3500V for 4 h, maximum to a total of 14000 Vh. The focused strips were either immediately run on a second dimension polyacrylamide gel or stored at -70^0C. The 2-D SDS-PAGE standard (Bio-rad, Hercules, CA, USA) was used for isoelectric point (pI) and molecular weight determination of the identified proteins on the 2D-PAGE gel.

2.4.2. Second Dimension

For the second dimension gel electrophoresis, the gel strips were incubated with equilibration buffer (0.5M Tris-HCl pH 8.8, 6M urea, 30% glycerol, 2% SDS, 1% DTT, 0.002% bromophenol blue) for 15 min. Strips were then incubated in the same equilibration buffer containing 2.5% iodoacetamide for an additional 15 min. Excess iodoacetamide was removed by soaking strips (1 min) in the Tris-glycine buffer (0.1% SDS, 25mM Tris base, pH 8.3, 192mM glycine) and placed on to a 12% polyacrylamide gel (Laemmli, 1970). Strips were overlaid with 0.5% low melting agarose sealing solution (0.5M Tris base, 0.1% SDS, 0.001% bromophenol blue). Electrophoresis was performed for 6 h at 30^0C, 130 V (SE 600 unit Hoefer Inc., San Franscisco, CA, USA).

2.4.3. 2D-Tris-tricine PAGE

To visualize low molecular weight proteins at a higher resolution in a 2D-PAGE, Tris-tricine gels were made according to Schägger (2006) with slight modifications in the protocol. The first dimension IEF and strip equilibration were executed as explained above. For second dimension, 16% Tris-tricine gels were prepared using (50% acrylamide, 33% resolving buffer, pH 8.45, 16.5% glycerol, 1.25% APS and 0.125% TEMED) and performed using cathode buffer (0.1M Tris, pH 8.9, 0.1M tricine, 0.1% SDS) and anode buffer (0.2M Tris, pH 8.9) at a constant voltage, 100 V, for 18 h. Low range rainbow marker (GE Healthcare, Uppsala, Sweden) was used for determination of the molecular weight of the identified protein spots.

The 2-D PAGE gels were visualized by staining with Coomassie Blue-R250, and documented using Alpha Innotech ChemImager (AlphaImager™ IS-4400, AlphaEaseFC™ 3.2.1, Alpha Innotech Corporation, San Leandro, CA, USA). For comparisons of photophase, scotophase and mated MAGs the samples were prepared and electrophoresed in a single run under the same experimental conditions (in triplicates). Gel images were analyzed using Compugen (Z3 2D-PAGE Analysis system, 3.0.5, Tel Aviv, Israel). The protein spot intensity levels were normalized between gels by dividing the spot intensity level by the sum of the intensities of all the spots in the gel. Spots of interest were excised and sent to Smoler Proteomics Center for identification by LC-MS. Protein identification was performed using NCBI data base.

RESULTS

3.1. Comparison of leached MAG proteins from males during the photophase and scotophase

Samples of proteins from male accessory glands (MAGs) were extracted into a physiological medium from naïve males during the scotophase and the photophase and a total of 98 proteins were identified and compared using LC-MS/MS out of which the levels of 52 were found to be up-regulated during the scotophase (Tables 1a-1h). Trypsin-digested protein fragments were sequenced and compared to the *Bombyx mori* (L.) (Lepidoptera: Bombycidae) and *D. melanogaster* protein data bases and similarities in the amino acid sequences were documented as percentage of protein homology. The proteins identified can be classified into 7 different functional groups: proteins involved in signal transduction; immune function; lipid transporting; gene function; cytoskeletal proteins; apoptosis; and metabolism; the rest of the proteins identified were of unknown function (Fig 1).

The majority of the identified proteins belong to the metabolism functional group (37), 13 were up-regulated during the scotophase and, in addition all the proteins, except 8, have 65% or more homology to proteins in the *B. mori* data base and 22 bear ≥81% homology to proteins in the *D. melanogaster* data base (Table 1a). The second largest group is the gene functional group, which includes 24 proteins, 18 of which

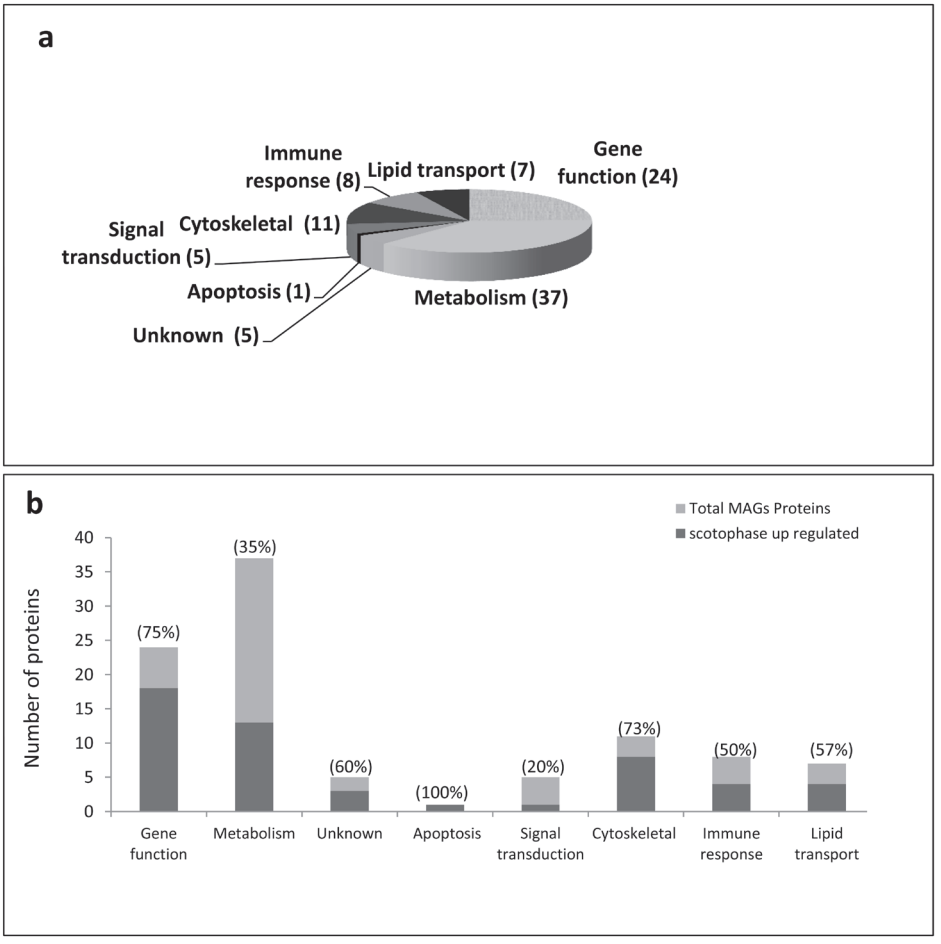

Figure 1: The classification of the proteins identified by LC-MS/MS into 7 different functional groups from samples of leached proteins of MAG incubations. (a): Number of proteins in each group. (b): The total number of proteins identified showing the percentage of up regulated proteins during the scotophase when compared to the photophase.

were up-regulated during the scotophase with 17 proteins bearing ≥86% homology to proteins in the *B. mori* data base and 12 proteins with ≥80% homology to proteins in the *D. melanogaster* data base (Table 1b). The third largest group is the cytoskeletal functional group which includes 11 proteins, 8 of which were up-regulated during the scotophase with 87% and 85% homology to proteins in the *B. mori* and the *D. melanogaster* respectively, with the exception of 4 unique proteins bearing no homology (Table 1c). In the immune functional group of 8 proteins, 4 of them were up-regulated during the scotophase with 9 proteins bearing 77% homology or more to proteins in the

Table 1a: LC-MS/MS identified proteins involved in metabolism

Protein name	Organism	NCBI protein accession #	Relative level of up-regulation during the scotophase	% homology to *B. mori*	% homology to *D. melanogaster*	P (pro)[1]
Glutathione S-transferase	*Corcyra cephalonica*	ABB29466.1	0.17	100	-	0.000234
Pyruvate carboxylase	*Glossina morsitans*	ADD19893.1	0.19	-	100 (RH5779)	3.63E-11
Sex-specific storage-protein 1 precursor	*Bombyx mori*	NP_001106747	0.21	85	-	3.18E-09
Short chain type dehydrogenase	*Aedes aegypti*	XP_001649973.1	0.25	-	-	0.000478
Pyruvate dehydrogenase E1 component beta subunit	*Bombyx mori*	NP_001040546.1	0.25	100	89	3.21E-09
Basic juvenile hormone-suppressible protein 2 precursor (sex-specific storage-protein 1 precursor)	*Helicoverpa armigera*	ABK29487.1	0.25	82	-	5.83E-10
Very methionine rich hexamerin precursor; VMtH (sex-specific storage-protein 1 precursor)	*Hyalophora cecropia*	AAB8647.1	0.26	76	-	2.05E-09
Methionine-rich storage protein	*Spodoptera exigua*	ABX55887.1	0.35	65	-	3.68E-07
Muscle glycogen phosphorylase	*Bombyx mori*	NP_001116811.1	0.39	100	94	9.91E-07
PREDICTED: similar to ATP-citrate synthase	*Tribolium castaneum*	XP_001808341.1	0.41	-	95	2.71E-07
Malate dehydrogenase	*Bombyx mori*	NP_001093280.	0.41	100	100	1.21E-11
Fructose 1,6-bisphosphate aldolase	*Bombyx mori*	NP_001091766.	0.42	100	94	8.54E-06
H+ transporting ATP synthase beta subunit isoform 2	*Bombyx mori*	NP_001041705.1	0.43	100	-	3.42E-09
Aldolase	*Drosophila melanogaster*	ABH06768.1	0.45	-	88	3.11E-09
Pyruvate kinase	*Bombyx mori*	NP_001036906.1	0.45	100	92	3.48E-08
Methionine-rich storage protein 1	*Manduca sexta*	AAA29321.1	0.54	82	-	1.85E-07
Vacuolar V-type H(+)-ATPase B subunit	*Helicoverpa armigera*	ADK94761.1	0.54	100	100	8.03E-06
Iron regulatory protein 1	*Manduca sexta*	AAK39637.1	0.59	-	84	1.18E-07
ATP synthase	*Bombyx mori*	NP_001040233.1	0.64	100	100	1.46E-09
Abnormal wing disc-like protein	*Bombyx mori*	NP_001093284.1	0.65	95	90	7.66E-10
Mitochondrial malate dehydrogenase	*Lysiphlebus testaceipes*	AAY63978.1	0.68	-	-	1.25E-06
ADP/ATP translocase	*Helicoverpa armigera*	AAP20934.2	0.76	94	100	4.17E-05
Aldehyde dehydroxygenase	*Heliothis virescens*	ACX53758	1	100	-	1.13E-05
NADH dehydrogenase subunit 1	*Biomphalaria pfeifferi*	AAP04228.1	Upregulated from zero value in photophase	-	-	0.000347

Table 1a: ***cont.***

Protein name	Organism	NCBI protein accession #	Relative level of up-regulation during the scotophase	% homology to *B. mori*	% homology to *D. melanogaster*	P (pro)[1]
Imaginal disc growth factor-like protein	*Mamestra brassicae*	ABC79625.1	1.02	93	-	1.32E-07
Transketolase	*Bombyx mori*	NP_001040158.1	1.1	100	90 (CG8036)	7.1E-07
Phosphoglyceromutase	*Bombyx mori*	NP_001037540.1	1.1	100	100	6.14E-13
Dihydrolipoamide dehydrogenase	*Bombyx mori*	NP_001037054.1	1.13	100	93 (CG7430)	5.73E-06
ATP synthase	*Helicoverpa zea*	ADJ95799.1	1.18	100	94	2.07E-06
Ecdysteroid-inducible angiotensin-converting enzyme-related gene product	*Bombyx mori*	NP_001036859.1	1.66	100	72	1.13E-08
Cytosolic malate dehydrogenase	*Bombyx mori*	NP_001040257	2.01	100	-	4.68E-11
Triosephosphate isomerase (TIM)	*Bombyx mori*	AAU84716	2.57	82	81	2.21E-11
Glyceraldehyde-3-phosphate dehydrogenase	*Bombyx mori*	ABX57319.1	4.04	100	-	0.000142
Glyceraldehyde-3-phosphate dehydrogenase	*Helicoverpa armigera*	AEB26314.1	0.75 - 5.24	92	100	2.11E-10
Nucleoside diphosphate kinase (NDK) (NDP kinase) (Abnormal wing disks protein)	*Bombyx mori*	NP_001093284	5.77	95	90	3.32E-07
Imaginal disk growth factor	*Bombyx mori*	BAF73623.1	18.74	95	85	4.13E-06
Fructose-bisphosphate aldolase	*Cerebratulus lacteus*	AAZ30645.1	70.7	-	-	0.000183

[1]Displays the probability of finding a match as good as or better than the observed match by chance.

=Up-regulation ≤1 =Up-regulation >1≤5 =Up-regulation >5≤20 =Up-regulation >20≤200

B. mori data base and 4 bearing a 75% homology or more to proteins in the *D. melanogaster* data base (Table 1d). In the lipid transporting functional group, consisting of 6 proteins, 4 were up-regulated during the scotophase with only 2 proteins bearing homology to proteins in the *B. mori* data base (79% and 91% homology respectively) and 3 proteins with ≥60% homology to the proteins in the *D. melanogaster* data base, the remaining two, which bear no homology to either *B. mori* nor *D. melanogaster*, do not have a known function (Table 1e). In the signal transduction functional group consisting of 5 proteins, 1 was up-regulated during the scotophase. Three of the proteins were 100% homologous to proteins in the *B. mori* and *D. melanogaster* data base and 1 protein was 83% homologous to *D. melanogaster* ortholog (Table 1f). Five proteins of unknown function included 3 proteins which were up-regulated during the scotophase. One of these proteins (a glycoprotein) is 60% homologous to a *B. mori* protein and 4 proteins are homologous to *D. melanogaster* proteins, 3 with 100% homology,

Table 1b: LC-MS/MS identified proteins with gene function

Protein name	Organism	NCBI protein accession #	Relative level of up-regulation during the scotophase	% homology to *B. mori*	% homology to *D. melanogaster*	P (pro)[1]
Elongation factor 1-alpha	*Bombyx mori*	NP_001037510.1	0.08	100	100	4.47E-06
Histone H2B-like protein	*Bombyx mori*	NP_001153668.1	0.56	94	-	4.18E-09
Eukaryotic translation initiation factor 4A	*Bombyx mori*	ABF51379.1	0.9	100	-	9.26E-05
Ribosomal protein S2	*Bombyx mori*	NP_001037564.1	0.9	100	85	0.000687
Elongation factor 1 alpha	*Siphona n. sp.*	AAM00346.1	0.97	-	-	6.07E-09
Translation elongation factor 2 isoform 1	*Bombyx mori*	NP_001037593.1	0.98	100	-	4.88E-07
EF-1-alpha	*Drosophila melanogaster*	CAA29993.1	1.02	-	85	0.000107
Elongation factor-1 alpha	*Oeconesus maori*	AAL34044	1.04	-	-	4E-06
Elongation factor 1 alpha	*Trichoplusia ni*	ABV68853.1	1.04	-	-	4E-06
Ribosomal protein L24	*Bombyx mori*	NP_001037231.1	1.04	100	80	6.49E-06
Ribosomal protein L11	*Helicoverpa armigera*	ABK29482	1.71	100	100	3.45E-08
Ribosomal protein P1	*Bombyx mori*	CAD35493	1.85	94	94	3.28E-05
histone H2A-like protein 2	*Drosophila melanogaster*	NP_524519.1	2.26	-	100	4.91E-05
histone H3	*Equus caballus*	XP_003365273.1	2.31	-	-	0.00083
Ribosomal protein L14	*Spodoptera frugiperda*	AAK92157	3.14	86	-	0.000148
Histone H4	*Pan troglodytes*	XP_003311105.1	3.82	-	-	0.000898
Ribosomal protein L27	*Bombyx mori*	NP_001037235	4.44	100	-	0.000487
poly A binding protein	*Bombyx mori*	NP_001091823.1	6.15	100	-	1.28E-05
Ribosomal protein L5	*Bombyx mori*	AAV34814	10.88	100	-	8.66E-08
Elongation factor 2	*Drosophila pseudoobscura*	NP_724357.1	11.79	86	93	4.88E-07
Ribosomal protein S3a	*Heliothis virescens*	AAK59927	17.68	100	82 (LD08549p)	7.3E-12
hnRNPA/B-like 28	*Bombyx mori*	ACM44033	28.28	100	100 (hrp48)	7.91E-09
Ribosomal protein P0	*Bombyx mori*	NP_001037123	28.28	93	92	4.97E-06
Ribosomal protein L7	*Bombyx mori*	NP_001037135	141.42	88	81	9.59E-06

[1]Displays the probability of finding a match as good as or better than the observed match by chance.

=Up-regulation ≤1 =Up-regulation >1≤5 =Up-regulation >5≤20 =Up-regulation >20≤200

one with a 53% homology (Table 1g). The apoptosis functional group is represented by CED-12, homologous to *Apis mellifera* (L.) (Hymenoptera: Apidae) CED-12 but bears no homology to either *B. mori* or *D. melanogaster* data base proteins (Table 1h). CED-12 was up-regulated during the scotophase.

Table 1c: LC-MS/MS identified proteins with cytoskeletal function

Protein name	Organism	NCBI protein accession #*	Relative level of up-regulation during the scotophase	% homolgy to *B. mori*	% homology to *D.melanogaster*	P (pro)[1]
Muscular protein 20	*Bombyx mori*	NP_001040476.1	0.43	93	-	8.03E-05
Actin-depolymerizing factor 1	*Bombyx mori*	NP_001093278.1	0.77	100	100	1.25E-05
Beta-tubulin	*Bombyx mori*	NP_001036965.1	0.83	100	100	0.000687
PREDICTED: similar to muscle protein 20-like protein	*Tribolium castaneum*	EFR23143.1	1.03	87	93	1.89E-06
Cytoplasmic actin	*Hirudo medicinalis*	BAH79732.1	1.1	-	-	2.15E-05
Alpha-tubulin	*Apis mellifera*	XP_391936	1.2	-	-	1.73E-08
Beta-1 tubulin	*Drosophila melanogaster*	AAA28989.1	1.2	-	100	4.09E-09
Actin	*Monodelphis domestica*	XP_001366709.2	1.2	100	-	6.56E-06
Beta-tubulin	*Bombyx mori*	NP_001036888.1	2.88	100	100	4.88E-08
Muscle protein 20, isoform A	*Drosophila melanogaster*	NP_476643	3.54	-	100	0.000205
PREDICTED: similar to Muscle protein 20 CG4696-PA, isoform A	*Apis mellifera*	XP_001120602.2	70.7	100	85	0.000153

[1]Displays the probability of finding a match as good as or better than the observed match by chance.

=Up-regulation ≤1 =Up-regulation >1≤5 =Up-regulation >5≤20 =Up-regulation >20≤200

Table 1d: LC-MS/MS identified proteins with immune function

Protein name	Organism	NCBI protein accession #	Relative level of up-regulation during the scotophase	% homology to B. mori	% homology to D. melanogaster	P (pro)1
Heat shock protein (hsp21.4)	Bombyx mori	NP_001036985.1	0.59	100	94	7.96E-05
PREDICTED: similar to cyclophilin-like protein isoform 1	Tribolium castaneum	XP_966308.1	0.63	88	-	3.25E-06
Putative peptidyl-prolyl cis-trans isomerase	Maconellicoccus hirsutus	ABM55516.1	0.63	93	-	0.000385
Defensin precursor	Spodoptera frugiperda	AAM96925.1	0.95	92	-	8.85E-05
Prophenoloxidase activating enzyme	Helicoverpa armigera	ABU98654.1	1.2	-	82	8.46E-08
Prophenoloxidase subunit 2	Helicoverpa armigera	AAZ52554.1	2.38	77	75	1.12E-06
Heat shock protein cognate 70	Helicoverpa armigera	AEB26315.1	3.98	100	100	1.41E-11
Heat shock protein 90	Helicoverpa armigera	ADD21559	10.68	-	-	0.000166

[1]Displays the probability of finding a match as good as or better than the observed match by chance.

=Up-regulation ≤1 =Up-regulation >1≤5 =Up-regulation >5≤20 =Up-regulation >20≤200

Table 1e: LC-MS/MS identified proteins with lipid transporting function

Protein name	Organism	NCBI protein accession #	Relative level of up-regulation during the scotophase	% homology to *B. mori*	% homology to *D. melanogaster*	P (pro)[1]
VHDL receptor	*Helicoverpa zea*	AAR37334.1	0.53	-	-	1.63E-08
Diazepam-binding inhibitor (ACBP)	*Helicoverpa armigera*	AAR37334.1	0.63	-	67	3.93E-09
Fatty acid-binding protein 3 (cellular retinoic acid binding protein)	*Helicoverpa armigera*	ACB54950.1	1.11	-	-	0.000691
Apolipophorin III	*Trichoplusia ni*	ABV68867	1.98	-	-	2.54E-07
Chemosensory protein 2	*Heliothis virescens*	AAV34687.1	47.14	91	63 (unnamed protein)	6.01E-05
Vitellogenin precursor	*Blattella germanica*	CAA06379.2	141.42	-	60	0.000646

[1]Displays the probability of finding a match as good as or better than the observed match by chance.

=Up-regulation≤1 =Up-regulation >1≤5 =Up-regulation >5≤20 =Up-regulation >20≤200

Table 1f: LC-MS/MS identified signal transduction proteins

Protein name	Organism	NCBI protein accession #	Relative level of up-regulation during the scotophase	% homology to B. mori	% homology to D. melanogaster	P (pro)1
Basic hexamerin. Insect storage protein receptor	Helicoverpa zea	AAR32137	0.4	-	-	1.09E-10
Ubiquitin-related modifier protein	Helicoverpa armigera	ADF30256.1	0.66	100	100	0.000914
GDP dissociation inhibitor	Drosophila melanogaster	AAA28567.1	0.87	-	100	9.01E-07
Transgelin	Bombyx mori	NP_001040372.1	0.9	100	100 (Chd64)	1.5E-06
14-3-3ZETA (small acid protein; regulator)	Helicoverpa armigera	ACS12990.1	4.44	100	83	8.43E-06

[1]Displays the probability of finding a match as good as or better than the observed match by chance.

=Up-regulation≤1 =Up-regulation >1≤5 =Up-regulation >5≤20 =Up-regulation >20≤200

Table 1g: LC-MS/MS identified proteins with unknown functions

Protein name	Organism	NCBI protein accession #*	Relative level of up-regulation during the scotophase	% homology to *B. mori*	% homology to *D. melanogaster*	P (pro)[1]
PREDICTED: similar to RH09070p	*Nasonia vitripennis*	XP_001601503	0.25	-	-	0.000478
CG11089, isoform A	*Drosophila melanogaster*	NP_651305.1	0.69	-	100	2.39E-07
CG1640-PA, isoform A	*Drosophila melanogaster*	NP_727696.2	1.12	-	100	2.39E-07
Unknown (27 kDa glycoprotein)	*Helicoverpa armigera*	ABU98620	2.74	61	53 (IP04208p)	1.69E-09
RH71862p	*Drosophila melanogaster*	AAM29636	2.8	-	100	2.5E-05

[1]Displays the probability of finding a match as good as or better than the observed match by chance.

=Up-regulation≤1 =Up-regulation >1≤5 =Up-regulation >5≤20 =Up-regulation >20≤200

Table 1h: LC-MS/MS identified proteins involved in apoptosis

Protein name	Organism	NCBI protein accession #*	Relative level of up-regulation during the scotophase	% homology to *B. mori*	% homology to *D. melanogaster*	P (pro)[1]
CED-12	*Apis mellifera*	XP_395913.3	3	-	-	1.97E-05

[1]Displays the probability of finding a match as good as or better than the observed match by chance.

=Up-regulation ≤1 =Up-regulation >1≤5 =Up-regulation >5≤20 =Up-regulation >20≤200

3.2. Effect of mating on MAG protein content

With the purpose of enlightening our knowledge as to the identity of the proteins that might be transferred from the male to the female during copulation, we performed comparative 2D electrophoresis using 12% SDS-PAGE as well as 16% Tris-tricine SDS-PAGE the latter gel for better resolution of the lower molecular weight proteins. For this purpose we focused on those proteins that were not only up-regulated during the scotophase when compared to the photophase, but also down-regulated in MAGs from mated males during the scotophase when compared to MAGs from naïve males during the scotophase. A total of 18 protein spots in the 2D 12% SDS-PAGE gels (Table 2) and 45 protein spots in the 2D 16% Tris-tricine SDS-PAGE

Table 2

Relative quantitative changes in protein expression using 2D 12% SDS-PAGE of MAG extracts in scotophase.

Spot No.	pI	M_r (kDa)	[1]Fold change in photophase	Fold change after mating
1	7.1	15.1	2.99	0.456
2	8.5	27.5	0.897	0
3	8.0	42.2	2.574	0
4	4.5	47.4	6.426	0
5	4.5	47.0	2.147	0
6	4.7	32.0	3.328	0.093
7	4.6	27.0	2.234	0.440
8	4.1	19.1	3.236	0
9	4.5	14.3	3.427	0
10	4.2	14.4	5.600	0
11	4.1	14.9	6.054	0
12	4.0	15.6	0	0
13	4.6	12.9	0	0
14	5.7	14.1	6.010	0
15	6.0	38.5	2.487	0
16	4.1	14.1	1.284	0.161
17	4.8	19.8	2.962	0.794
18	4.4	17.1	3.352	0.206

[1]Levels < 1 indicate up-regulation during the scotophase relative to the photophase and depletion during the scotophase. Shaded rows indicate spots that correspond to both, up-regulation during the scotophase and depletion after mating.

Table 3

Relative quantitative changes in protein expression using 2D 16% Tris-tricine SDS-PAGE of MAG extracts

Spot No.	pI	M_r (kDa)	[1]Fold change in Photophase	Fold change after Mating	NCBI Blast similarities
19	8.0	42.2	1.513	0.128	
20	7.2	37.9	0.555	0	
21	8.5	27.5	0.171	0	
22	7.6	24.2	0.276	0	
23	9.4	14.9	0	0	Acyl CoA binding protein (ACBP)
24	8.2	11.0	0.080	0	
25	7.6	10.8	0.126	0	
26	4.9	10.2	0.122	0.103	Cecropin [Helicoverpa armigera]
27	5.2	10.7	1.364	0	
28	5.5	11.4	0.077	0	Protease associated transposable elements
29	5.7	11.3	0.455	0.104	Protease associated transposable elements
30	5.7	13.2	0.289	0.271	
31	5.8	13.9	0.886	0	
32	5.3	13.4	1.108	0.106	
33	5.1	12.4	1.137	0.092	
34	4.8	11.1	1.846	0	
35	4.8	12.7	0.867	0	
36	4.5	15.1	1.234	0.844	
37	4.9	14.5	1.338	0	
38	5.1	14.5	1.003	0	
39	4.9	16.3	1.310	0	
40	5.6	16.2	0	0	
41	5.3	17.2	1.150	0.210	
42	4.7	32.0	1.371	0	
43	6.0	38.5	1.320	0.129	
44	3.9	29.9	0	0.178	
45	3.9	24.9	1.830	0	
46	6.1	26.6	0.375	0	
47	6.7	26.9	0.258	0	
48	9.2	22.7	0.050	0.193	
49	6.9	16.3	0.185	0	
50	7.0	17.0	0.230	0	
51	7.0	17.9	0.329	0	
52	6.2	18.0	0	0	
53	8.1	10.0	0	0	
54	5.7	12.4	2.118	1.103	
55	9.2	14.0	0.052	0	Acyl CoA binding protein (ACBP)
56	4.9	11.6	0.355	0	
57	5.5	10.9	0	0	
58	6.4	12.8	0	0	
59	7.1	12.6	0	0.792	
60	7.2	13.3	0	0.798	Regulator of G protein signaling domain
61	7.4	13.4	0	0.223	
62.	5.2	39.8	0.807	0.477	
63.	4.3	45.2	0	0.434	
64.	6.6	11.7	1.124	0	

[1]Levels < 1 indicate up-regulation during the scotophase relative to the photophase and depletion during the scotophase.
Shaded rows indicate spots that correspond to both, up-regulation during the scotophase and depletion after mating.

gels (Table 3; Fig 2a-c) exhibited changes during the photoperiod and after mating respectively. Two protein spots in the 2D 12% SDS-PAGE separation and 31 protein spots in the 2D 16% Tris-tricine SDS-PAGE separation corresponded to proteins that were up-regulated during the scotophase and concomitantly down-regulated af-

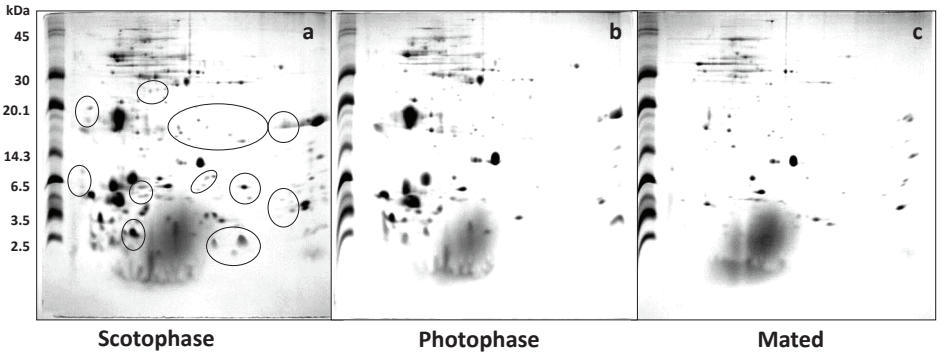

Figure 2: A typical example of a comparison between MAG protein profiles from (a) naïve males during the scotophase, (b) naïve males during the photophase and (c) mated males using two-dimensional 16% Tris-tricine SDS-PAGE. Indicates areas in which spots are up-regulated during the scotophase and depleted after mating.

ter mating. Five of these latter spots were successfully identified through LC-MS sequencing resulting in the identification of an acyl CoA binding protein (ACBP), Cecropin, protease associated transposable element (corresponding to 2 spots) and a G-protein coupled signaling domain protein (Table 3).

DISCUSSION

Proteomics is recognized as one of the main perspectives of science in the post genomic era. A proteomic approach coupled with genetic resources could be used to unravel additional components and gene functions that support the complex biological processes involved in mating and post mating behaviors of *H. armigera*. The role of MAG secretions in inducing female non receptivity has been researched extensively in various insects. Progress in this field of interest in the moth *H. armigera* may contribute a great deal to finding an alternative solution in managing the moth's population. In this report we identify some MAG proteins in the seminal fluid secretion that undergo changes in accordance to photoperiod and are reduced or depleted after mating, thereby having a functional significance during mating. Assuming that the proteins that are up-regulated during the scotophase are most likely produced in readiness to mating, initially LC-MS/MS identified leached proteins from MAGs during the photophase were compared to MAGs during the scotophase. Changes in MAG content were further studied using 2D SDS PAGE to identify extracted proteins that are both up-regulated during the scotophase and depleted after mating and thus may be transferred to the female during copulation. Interestingly, different candidates are revealed by the two approaches with no overlap. This may be accounted for by the varied sensitivities of the two methods.

More than half of the identified proteins were up-regulated during the scotophase with most at a level up to 5 fold but 7 of the proteins were up-regulated to levels of more than 20 fold. Each protein was compared to the *B. mori* and the *D. melanogaster* protein databases. The comparison to *B. mori* was performed due to the phylogenetic proximity of these two moth species and because a variety of *H. armigera* proteins show high homology to *B. mori* proteins (Liubin *et al*., 1999; Rafaeli, 2002; 2009; Teese *et al*., 2010). The synthetic *Drosophila* peptides, *Drm*SP and DUP99B were shown to be functional in *H. armigera* (Fan *et al*., 1999; 2000) thus we also compared the identified proteins to the *D. melanogaster* protein data base with the purpose of revealing possible functional characteristics to the identified *H. armigera* proteins. Indeed, most of the identified *H. armigera* proteins bore high levels of homologies to proteins in both the *B. mori* and the *D. melanogaster* databases.

When comparing the functions of *Drosophila* seminal fluid proteins we identified many functional similarities thus implying that the roles of seminal fluid proteins are conserved throughout the Insecta as previously claimed (Avila *et al*., 2011). One of the highly up-regulated proteins included vitellogenin precursor, whose presence in male accessory glands has not been shown to date. However, the peptide vitellogenin precursor was reported in the cockroach *Blattella germanica* (L.) (Blattodea: Blatellidae) and in *Anopheles gambi*ae (Giles) (Diptera: Culicidae) as a lipid transporter (Rogers *et al*., 2008) and it could be speculated that in this case the peptide may have a similar function mediating lipid-transport from the male to the female during copulation, or facilitating their transport within the female after transfer. No doubt, verification of this function must await further investigations.

Another protein that was observed to be highly up-regulated (47 fold) during the scotophase is the chemosensory protein 2, belonging to a class of small (10-15 kDa), soluble proteins secreted into the sensillar lymph of insect chemosensory sensilla. These proteins bind semiochemicals such as pheromones and odor molecules and deliver those molecules in the aqueous lymph to olfactory receptors (Pelosi *et al*., 2006). In the past few years many chemosensory proteins have been identified in insect contact and olfactory sensilla with odorant transport function, but they have also been found in non-sensory tissues such as the regenerating legs of *Periplaneta americana* (L.) (Blattodea: Blattidae) (Kitabayashi *et al*., 1998; Nomura *et al*., 1992) and in the antennae and pheromone glands of *Mamestra brassica* (L.) (Lepidoptera: Noctuidae) (Jacquin-Joly *et al*., 2001), suggesting that these proteins may be involved in transport of hydrophobic molecules through different aqueous media. Moreover, several of the possible *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae) seminal fluid proteins are predicted as pheromone/odorant binding proteins (South *et al*. 2011). In *D. melanogaster* and *Aedes aegypti* (L.) (Diptera: Culicidae) odorant binding proteins are also identified as a class of putative seminal fluid proteins (Findlay *et al*., 2008; Sirot *et al*., 2008). The functional significance of these proteins in reproduction has yet to be determined, but work suggests that odorant molecules play a chemo-attractant role for sperm (Fukuda *et al*., 2004).

An additional protein identified in this study with lipid transporting function, is

apolipoprotein, a glycoprotein bound to serum lipid bodies, also found in *A. mellifera* seminal fluid proteins and reported to be linked to sperm capacitation in mammals (Baer *et al*., 2009). Another identified protein belonging to this group is the acyl-CoA-binding protein (ACBP), which is known in female moths as a lipid transporter and in assisting in the pheromone biosynthetic pathway (Itagaki and Conner, 1986; Matsumoto *et al*., 2001; Ohnishi *et al*., 2006). There has been no evidence up until the present study for its presence or its function in male reproductive organs. Indeed, future studies are needed in order to decipher its role in the males.

The up-regulation of several ribosomal proteins observed in the present study may be a reflection of induced levels of proteins synthesis. Upon repeated mating the male replenishes sperm and seminal fluid supplies to maintain a high level of fertility (Hihara, 1981). Herndon *et al*. (1997) showed stimulation of some seminal fluid proteins as a result of Juvenile Hormone analog and/or 20-hydroxyecdysone application on the cuticle of male *Drosophila* and that prevention of seminal fluid transfer to females inhibited this stimulation. Indeed, we show here that enhanced protein synthesis of some of the seminal fluid proteins occurs during the scotophase. This enhanced synthesis may involve the same mechanisms that occur after mating to replenish stocks, but this hypothesis awaits further examination.

Heat shock proteins, Hsp70 and Hsp90 were observed to be up-regulated in *H. armigera* MAGs during the scotophase by 4 and 10 fold respectively. These represent immune functional proteins which are usually expressed and/or utilized during stress conditions to repair or protect nuclear proteins and minimize protein aggregation, thereby preventing genetic damage. There is evidence that Hsp90 is present in the seminal fluid of *A. mellifera* (Baer *et al*., 2009) and *A. gambiae* (Dottorini *et al*., 2007); however, it was shown to play an important role in signal transduction in its association with the ecdysone receptor/ultraspiracle complex, where its presence is essential for the proper functioning of the complex (Arbeitman and Hogness 2000). Thus Hsp90 may well act as a component of ribosomal protein regulation which was shown to be regulated by ligand binding to the ecdysone receptor (Herndon *et al*., 1997). Other functions attributed to heat shock proteins include a chaperone role in the selective stability of some proteins in the seminal fluid (Baer *et al*., 2009), and as an intercellular signal facilitating sperm-egg interactions (Asquith *et al*., 2005). However, there is no evidence that these proteins act as chaperones outside the cell, thus the possibility that their presence in the seminal fluid is due to ruptured cells cannot be overlooked.

The prophenoloxidase activating enzyme and prophenoloxidase subunit 2, which are classified as part of the immune functional group, were observed to be up-regulated during the scotophase by 1.2 and 2.38 respectively. They represent a significant component of the innate immune response of arthropods, activation of which leads to melanin synthesis and the assembly of antimicrobial proteins through the stimulation of the Toll signaling pathway (El Chamy *et al*., 2008; Ferrandon *et al*., 2007). The activity of the antimicrobial proteins during copulation is vital, because mating has the potential of introducing a variety of pathogens into the female reproductive tract that could negatively influence the reproductive success of both sexes. In *D. melan-*

ogaster, several seminal fluid proteins have been found to have either indirect antimicrobial activity by modulating a female's ability to fight infection or direct activity by protecting gametes and zygotes (Lung and Wolfner, 2001; Mueller *et al.,* 2007; Samakovlis *et al.,* 1991; Wolfner, 2009). In addition, putative seminal fluid proteins in both *A. gambiae* and *A. aegypti* suggest that these proteins may also play a role in the immune response (Rogers *et al.,* 2008; Sirot *et al.,* 2008). South *et al.* (2011), using a proteomic based method, identified in *Tribolium* a putative predicted prophenoloxidase seminal fluid protein which is possibly involved in an immune response. Cecropin, identified in the MAGs through 2D SDS-PAGE, was also observed to be up-regulated during the scotophase and down-regulated after mating, thereby indicating a possible transfer to the female reproductive tract. Cecropins are potent antibacterial proteins that constitute a main part of the cell-free immunity of insects (Hultmark *et al.*, 1982) and may, thus, be beneficial to the mated female that might have been compromised during copulation.

The metabolic functional group, which includes the largest number of identified protein in the present study, is also known to represent a large part of the seminal fluid proteins in *Drosophila mojavensis* (Patterson *et al.*) (Diptera: Drosophilidae) male accessory glands, comprising almost 8% of candidate seminal fluid proteins (Kelleher *et al.*, 2009). However, this class of enzymes were not prominent amongst *D. melanogaster* seminal fluid proteins (Findlay *et al.*, 2008), nor amongst secreted proteins expressed in *D. melanogaster* male accessory glands (Swanson *et al.*, 2001), pointing to an important biological difference between these two species. Whether metabolic processes are specific to the male accessory gland, or they occur in female reproductive tracts, or both, remains unknown.

In the signal transduction group, 14-3-3 ZETA belonging to a family of conserved regulatory proteins that are expressed in all eukaryotic cells was the only protein showing up-regulation during the scotophase. These proteins have the ability to bind a multitude of functionally diverse signaling proteins, including kinases, phosphatases, and transmembrane receptors (Aitken *et al.*, 1992), thus they could have various functions in the reproductive process. According to the Fly-base data they are also expressed in *Drosophila* male accessory glands, but their role during copulation is unknown and awaits further research. Nonetheless, they too may reflect the presence of lysed cells.

A range of structural components are present in the male accessory glands and are up-regulated during the scotophase namely the muscle protein tubulin and actin. These proteins are most likely associated dynamically with sperm, as suggested by Baer *et al.* (2009) who also identified structural components in the *A. mellifera* seminal fluids. However their specific role/s in the reproductive system of males is yet to be deciphered.

CED-12 is the only peptide identified in this study which belongs to the apoptosis functional group and it is up-regulated during the scotophase compared to the photophase by 3 fold. It is a *Caenorhabditis elegans* (Maupas) (Rhabditida: Rhabditidae) – ortholog of the ELMO family of mammalian adaptor proteins involved in promoting

cell migration and phagocytosis of apoptotic cells (Gumienny *et al*., 2001). Its presence in other insect seminal fluid or its function in the reproductive process has not been reported to date.

In our study we have not been able to identify homologs to the three peptides considered to be very important in eliciting increased fecundity and in causing non-receptivity after mating (sex peptide, ovulin and DUP). This could be as a result of their relative expression at low levels and, therefore, swamping by the presence of other proteins expressed at higher levels. On the other hand, and despite the identification of positive immunoreactivity to sex-peptide (Nagalakshmi *et al*., 2004; 2007), these may differ in sequence such that homologies in short sequence fragments could not be identified using LC-MS/MS.

Although it is clear that seminal fluid proteins are among the most rapidly evolving proteins (Swanson and Vacquier, 2002), and their primary sequences evolve fast, the protein classes represented in seminal fluids are relatively conserved (Mueller *et al*., 2005). In addition, examining the seminal fluid of *Drosophila* species reveals rapid gain/loss of accessory glands protein genes to be a common attribute of *Drosophila* seminal fluid evolution (Findlay *et al*., 2008; Mueller *et al*., 2005), nevertheless, even as much of the knowledge of insect seminal fluid proteins has been acquired by studies in *Drosophila* species, attempts to identify homologs of *D. melanogaster* seminal fluid proteins outside of *Drosophila* have had limited success, most likely because of these rapid rates of evolution. For example, a recent study showed that *A. mellifera* seminal fluid proteins had more sequence similarities with human seminal fluid proteins than with *D. melanogaster* seminal fluid proteins (Baer *et al*., 2009). The lack of information about these proteins in key taxonomic groups including *H. armigera* currently limits our understanding of their functional significance. In order to broaden the knowledge in this field of research one possible way is to knockdown individual seminal fluid proteins, thereby revealing their role in the reproductive process. Further exploration of the *H. armigera* male reproductive proteins that were identified in this work, particularly those that are depleted during mating, will provide insight into their individual roles in the mating process.

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Supplement Fig 1:
Sequences of fragmented proteins obtained through LC/MS/MS of MAG extracts

Scan (s)	Peptide Sequence (matching searched sequences; differential modification indicated by * # according to SEQUEST)
AF429977_1 ribosomal protein S3A [Spodoptera frugiperda]	
3267	K.RQVGTTLVNR.T
11623	R.VFEVSLADLQADTDAER.S
RL11_SPOFR 60S ribosomal protein L11	
8123	K.V]LEQLTGQQPVFSK#.A
8131	K.VLEQLTGQQPVFSK.A
acidic p0 ribosomal protein [Carabus granulatus]	
12085	K.T]SFFQAXSIPTK#.I
12103	K.TSFFQAXSIPTK.I
AGAP003592-PA [Anopheles gambiae str. PEST]	
12085	-.T]SFFQALSIPTK#.-
12103	-.TSFFQALSIPTK.-
putative ribosomal protein L7 [Sipunculus nudus]	
11521	R.L]AEPYLAWGYPNLK#.S
11523	R.LAEPYLAWGYPNLK.S
ribosomal protein L7 [Spodoptera frugiperda]	
11521	-.I]AEPYIAWGYPNLK#.-
11523	-.IAEPYIAWGYPNLK.-
60S acidic ribosomal protein P1 [Plutella xylostella]	
12501	K.A]ANVDVEPYWPGLFAK#.A
12503	K.AANVDVEPYWPGLFAK.A
AF400185_1 ribosomal protein L14 [Spodoptera frugiperda]	
4797	R.ALVDGPCSGVSR.Q
10720	K.LVSVVDVIDQTR.A
Ribosomal protein L27 [Plutella xylostella]	
4294	K.VVNYNHLM*PTR.Y
GA19229-PA [Drosophila pseudoobscura]	
5252	-.GTGIVSAPVPK.-
5294	-.GSTGTLGNFAK.-
ribosomal protein L5 [Bombyx mori]	
4839	R.LSNKDVTCQVAYSR.I
7041	R.IEGDHIVCAAYSHELPR.Y
GA18066-PA [Drosophila pseudoobscura]	
4974	-.MKETAEAYLGK.-
8999	-.IINEPTAAAIAYGLDKK.-
9383	R.ARFEELNADLFR.S
GA17988-PA [Drosophila pseudoobscura]	
3571	K.M*KETAEAYLGKK.V
3593	K.IVITNDQNR.L
4148	K.MKETAEAYLGKK.V
8440	R.ITPSYVAFTADGER.L
8460	R.I]TPSYVAFTADGER.L
GRP78_APLCA 78 kDa glucose-regulated protein precursor (GRP 78) (BiP) (Protein 1603)	
8820	R.AKFEELNM*DLFR.S
10640	R.AKFEELNMDLFR.S
10653	R.A]K#FEELNMDLFR.S
GA11622-PA [Drosophila pseudoobscura]	
6172	-.H]SQFIGYPIK#.-
6269	-.HSQFIGYPIK.-
9439	K.GVVDSEDLPLNISR.E

heat shock protein [Antheraea yamamai]	
4915	-.H]IYYITGENR.-
4938	-.HIYYITGENR.-
8518	-.ALLFVPR.-
8637	-.RAPFDLFENK.-
82 kDa heat shock protein [Drosophila pseudoobscura bogotana]	
6172	K.H]SQFXGYPIK#.L
6269	-.HSQFXGYPIK.-
HSP83_DROPB Heat shock protein 83 (HSP 82)	
6172	-.H]SQFIGYPXK#.-
6269	-.HSQFIGYPXK.-
triosephosphate isomerase [Helicoverpa armigera]	
3842	K.TASPQQAQDVHASLR.N
3864	K.T]ASPQQAQDVHASLR.N
4213	R.I]QYGGSVTGANAK#.E
8760	R.N]WLSANASPDVAASVR.I
cytosolic malate dehydrogenase [Bombyx mori]	
3067	-.KMSSALSAAK.-
3859	R.IFKEQGQALDK.V
4761	R.IFKEQGQALDKVAR.K
6764	R.WVSM*GVVSDGSYGTPR.D
8762	K.V]LVVGNPANTNALICSK#.Y
8766	-.VLVVGNPANTNALICSK.-
PREDICTED: similar to Chd64 CG14996-PB [Apis mellifera]	
6604	R.A]GQGVISLQYGSNK#.G
12282	R.TLGVPAQETFQTVDLWER.Q
unknown [Helicoverpa armigera]	
4489	R.TCAENIKEGFK.S
7215	A.EEFNLPEDKAAQLR.A
8341	K.SLFDMETLKK.E
8351	K.S]LFDMETLK#K#.E
10910	R.IALFIAEGGPQCFQSK.A
10995	R.I]ALFIAEGGPQCFQSK#.A
11611	K.SCIYNLLDGVSPCVDGNM*R.D
12089	K.SCIYNLLDGVSPCVDGNMR.D
GA10287-PA [Drosophila pseudoobscura]	
10637	K.VFLGGLPSNVTETDLR.T
10638	K.V]FLGGLPSNVTETDLR.T
ecdysteroid-inducible angiotensin-converting enzyme-related gene product [Bombyx mori]	
8756	K.MFQM*SDEFFR.S
8795	K.M]FQM*SDEFFR.S
9401	K.M*GSSKPWPDAMEALTGQR.E
9868	K.M]GSSK#PWPDAMEALTGQR.E
10552	K.M*FQMSDEFFR.S
10834	K.L]WEDVK#PLYQQLHAYVR.K
10881	K.LWEDVKPLYQQLHAYVR.K
11013	G.WQDFQDFTLR.R
11198	K.MFQMSDEFFR.S
GA17427-PA [Drosophila pseudoobscura]	
4793	-.M*REIVHLQAGQCGNQIGAK.-
5512	-.EIVHLQAGQCGNQIGAK.-
6066	R.IM*NTYSVVPSPK.V
6944	R.I]MNTYSVVPSPK#.V

9771 R.Y]LTVAAVFR.G
11694 K.N]SSYFVEWIPNNVK#.T
11700 K.NSSYFVEWIPNNVK.T
RH71862p [Drosophila melanogaster]
9404 -.RINYQPPTVVPGGDLAK.-
9412 -.R]INYQPPTVVPGGDLAK#.-
apolipophorin III [Trichoplusia ni]
10737 P.SPLQDIEKHAAEFQK.T
10802 P.PSPLQDIEK#HAAEFQK#.T
10802 -.P]SPLQDIEKHAAEFQK#.-
11415 P.PSPLQDIEKHAAEFQK.T
alpha-Tubulin at 84B CG1913-PA [Drosophila melanogaster]
7230 K.YMACCMLYR.G
8359 R.Q]LFHPEQLITGK#EDAANNYAR.G
8380 R.QLFHPEQLITGKEDAANNYAR.G
9404 -.VGINYQPPTVVPGGDLAK.-
9412 K.V]GINYQPPTVVPGGDLAK#.V
10499 R.AVCM*LSNTTAIAEAWAR.L
11147 R.I]HFPLVTYAPVISAEK#.A
11179 R.IHFPLVTYAPVISAEK.A
11602 -.AVFVDLEPTVVDEVR.-
12064 R.TIQFVDWCPTGFK.V
12071 R.T]IQFVDWCPTGFK#.V
12506 R.L]IGQIVSSITASLR.F
13616 R.F]DGALNVDLTEFQTNLVPYPR.I
beta-Tubulin at 56D CG9277-PB, isoform B [Drosophila melanogaster]
4793 -.M*REIVHIQAGQCGNQIGAK.-
5512 -.EIVHIQAGQCGNQIGAK.-
5845 R.INVYYNEASGGK.Y
6866 R.INVYYNEASGGKYVPR.A
9924 R.I]SEQFTAMFR.R
9927 R.ISEQFTAMFR.R
NDKA_DROME Nucleoside diphosphate kinase (NDK) (NDP kinase) (Abnormal wing disks protein) (Killer of prune protein)
7739 R.QM*LGATNPADSLPGTIR.G
8755 R.Q]MLGATNPADSLPGTIR.G
8771 R.QMLGATNPADSLPGTIR.G
glyceraldehyde 3-phosphate dehydrogenase [Gammarus pulex]
6940 -.IVSNASCTTNCLAPIAK.-
6941 -.I]VSNASCTTNCLAPIAK#.-
glyceraldehyde-3-phosphate dehydrogenase [Plutella xylostella]
3902 R.LGKPATYDAIKQK.V
4672 R.LGKPATYDAIK.Q
elongation factor 2 [Culex pipiens quinquefasciatus]
13935 K.E]GIPDLSQYLDK#L.-
13963 K.EGIPDLSQYLDKL.-
elongation factor 1 alpha [Chlosyne acastus]
6311 -.I]GGLGTVPVGR.-
8562 -.A]LRXPLQDVYK#.-
8566 -.ALRXPLQDVYK.-
AF436631_1 elongation factor-1 alpha [Dolophilodes distinctus]
11745 -.V]ETGILK#PGTIVVFAPANLTTEVK#.-
11762 -.VETGILKPGTIVVFAPANLTTEVK.-
11745 -.V]ETGILK#PGTIVVFAPANITTEVK#.-

11762	-.VETGILKPGTIVVFAPANITTEVK.-
prophenoloxidase subunit 2 [Helicoverpa armigera]	
3911	-.FTHLNNRPFR.-
3954	R.DLSVQSNDPR.R
3955	-.D]LSVQSNDPR.-
5638	R.Y]VINVNNTGM*AR.R
5656	R.YVINVNNTGM*AR.R
5888	R.N]LAWALSDHRK#.M
5909	R.NLAWALSDHRK.M
6067	R.ETAAVIPQNVPR.T
6327	K.FM*DSQVFQQAR.E
6854	R.Y]VINVNNTGMAR.R
7289	R.NLAWALSDHR.K
7905	K.ALFQLTEK.L
11144	R.LSTESSVTIPFEQTFR.D
prophenoloxidase activating enzyme [Helicoverpa armigera]	
4153	R.KGTTSYQCGGVLINHR.Y
10560	R.YTYYVQPICLVDNR.V
imaginal disk growth factor [Bombyx mori]	
6406	-.GLCTGDKYPILR.-
6415	-.G]LCTGDK#YPILR.-
7326	-.S]TWGSLWHGIK#K#.-
8796	-.STWGSLWHGIK.-
imaginal disc growth factor 2 [Glossina morsitans morsitans]	
6406	R.GLCTGDKYPLLR.A
6415	R.G]LCTGDK#YPLLR.A
Actin 87E CG18290-PA, isoform A [Drosophila melanogaster]	
2599	R.H]QGVMVGM*GQK#.D
3037	R.HQGVM*VGMGQK.D
3037	-.HQGVMVGM*GQK.-
3560	R.H]QGVMVGMGQK#.D
3568	R.HQGVMVGMGQK.D
5306	R.GYSFTTTAER.E
5327	R.G]YSFTTTAER.E
6046	K.I]WHHTFYNELR.V
6048	K.IWHHTFYNELR.V
6672	-.A]VFPSIVGRPR.-
6725	-.AVFPSIVGRPR.-
6924	K.EITALAPSTIK.I
7929	R.VAPEEHPVLLTEAPLNPK.A
cytoplasmic actin [Hirudo medicinalis]	
6672	R.A]VFPSLVGRPR.H
6725	R.AVFPSLVGRPR.H
PREDICTED: similar to Muscle protein 20 CG4696-PA, isoform A [Apis mellifera]	
5170	Q.T]VIGLQAGSNK#.G
5172	Q.TVIGLQAGSNK.G
GA18362-PA [Drosophila pseudoobscura]	
2760	-.GATQAGQNLGAGR.-
5172	-.TIVGLQAGSNK.-
AGAP007643-PB [Anopheles gambiae str. PEST]	
4903	R.YLAEVATGETR.H
4918	R.Y]LAEVATGETR.H
GA19329-PA [Drosophila pseudoobscura]	
3056	R.YASICQSQR.I

3059	R.Y]ASICQSQR.I
4569	G.IAQAIVAPGK.G
4573	G.I]AQAIVAPGK#.G
8669	-.RPWALTFSYGR.-
8752	-.R]PWALTFSYGR.-
9973	R.IVPIVEPEVLPDGDHDLDR.A
PREDICTED: similar to poly A binding protein, cytoplasmic 1 isoform 2 [Tribolium castaneum]	
2557	R.KAHLTSQYM*QR.M
3255	R.KAHLTSQYMQR.M
fructose-bisphosphate aldolase [Cerebratulus lacteus]	
10195	-.GHTPSHLAM*LENANVLAR.-
12157	-.GHTPSHLAMLENANVLAR.-
PREDICTED: similar to Ced-12 CG5336-PA [Apis mellifera]	
7073	E.I]LELIQQQR.L
7085	E.ILELIQQQR.L
3021	R.YKDKIEAVKGQ.-
8168	-.AYVDCLLDR.-
glyceraldehyde-3-phosphate dehydrogenase [Bombyx mori]	
3902	-.LGKPASYEAIKQK.-
4909	-.GAQQNIIPASTGAAK.-
glyceraldehyde-3-phosphate dehydrogenase [Colias eurytheme]	
6940	K.VISNASCTTNCLAPLAK.V
6941	K.V]ISNASCTTNCLAPLAK#.V
10883	-.V]PVPNVSVVDLTVR.-
NADH dehydrogenase subunit 1 [Biomphalaria pfeifferi]	
13683	-.S]NKILFMIAPIM*GFGLAL.-
vitellogenin [Blattella germanica]	
4855	-.AVEHLLSTR.-
11213	-.QKGM*LVSHINVTVERK.-
His2A:CG31618 CG31618-PA [Drosophila melanogaster]	
7341	-.AGLQFPVGR.-
7362	R.A]GLQFPVGR.I
chemosensory protein 2 [Heliothis virescens]	
histone H3 [Tetrastemma elegans]	
4233	R.Y]RPGTVAXR.E
4247	R.YRPGTVAXR.E
AGAP012709-PA [Anopheles gambiae str. PEST]	
4233	-.Y]RPGTVALR.-
4247	-.YRPGTVALR.-
Histone H4 replacement CG3379-PC, isoform C [Drosophila melanogaster]	
7189	-.ISGLIYEETR.-
8631	K.VFLENVIR.D
cellular retinoic acid binding protein [Plutella xylostella]	
6044	K.SVCTFEGNTLK.Q
6096	-.A]IGVGLITRK#.-
6099	-.AIGVGLITRK.-
7453	-.A]IGVGLITR.-
7454	-.AIGVGLITR.-
GA20345-PA [Drosophila pseudoobscura]	
2963	R.VCHAHPTCAEALR.E
2968	-.VCHAHPTCAEALR.-
7514	K.V]GK#FPFLANSR.A

7521 K.VGKFPFLANSR.A
transketolase [Bombyx mori]
3837 -.NSTFSDKLR.-
4693 R.IDSIVATNASK.S
6969 K.L]RIDSIVATNASK#.S
elongation factor-1 alpha [Micrutalis calva]
11745 -.V]ETGXLK#PGXVVTFAPANLTTEVK#.-
11762 -.VETGXLKPGXVVTFAPANLTTEVK.-
CG1640 CG1640-PA, isoform A [Drosophila melanogaster]
9285 R.ALVVINPGNPTGQVLTR.E
9290 R.A]LVVINPGNPTGQVLTR.E
11294 -.V]FHAEFM*KKY.-
GA14392-PA [Drosophila pseudoobscura]
2658 R.HGESEWNQK.N
4757 -.ILIAAHGNSLR.-
4767 R.H]YGGLTGLNK#AETAAK#.Y
4777 R.HYGGLTGLNKAETAAK.Y
7896 K.Y]GEAQVQIWR.R