Apidologie 40 (2009) 481–495

DOI: 10.1051/apido/2009009

Available online at:
www.apidologie.org

Original article

Differential gene expression profiling in mucus glands of honey bee *(Apis mellifera)* drones during sexual maturation*

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Received 28 April 2008 – Revised 1 December 2008 – Accepted 20 January 2009

Abstract – The mating sign that each drone leaves when mating with a queen essentially consists of mucus gland proteins. We employed a Representational Difference Analysis (RDA) methodology to identify genes that are differentially expressed in mucus glands during sexual maturation of drones. The RDA library for mucus glands of newly emerged drones was more complex than that of 8 day-old drones, with matches to 20 predicted genes. Another 26 reads matched to the *Apis* genome but not to any predicted gene. Since these ESTs were located within ORFs they may represent novel honey bee genes, possibly fast evolving mucus gland proteins. In the RDA library for mucus glands of 8 day-old drones, most reads corresponded to a capsid protein of deformed wing virus, indicating high viral loads in these glands. The expression of two genes encoding venom allergens, *acid phosphatase-1* and *hyaluronidase*, in drone mucus glands argues for their homology with the female venom glands, both associated with the reproductive system.

male accessory gland / Representational Difference Analysis / honeybee / reproduction / deformed wing virus

1. INTRODUCTION

The major constituents of accessory gland secretions of insect males (MAGs) are carbohydrates, proteins, and lipids (Gillott, 2003). These are transferred to the female during copula, either as part of the spermatophore or in the form of seminal fluid. Whereas carbohydrates and lipids are important to sperm energy metabolism (Collins et al., 2006), the accessory gland proteins (Acps) are functionally more complex. Their functions have been studied in mated *Drosophila melanogaster* females where they enhance egg production, increase rates of ovulation, reduce sexual receptivity to other males, assist in the storage of sperm in the spermatheca, and may cause a reduction in the female's life span (Chen et al., 1988; Chapman et al., 1995; Neubaum and Wolfner, 1999). Acp-encoding genes were identified in differential hybridization screens, functional assays, and via an accessory gland EST screen (Schäfer, 1986; Monsma and Wolfner, 1988; Simmerl et al., 1995; Wolfner et al., 1997). In a particularly extensive screen, 57 new candidate Acps were identified from partial-gene sequencing of ESTs obtained from a *D. simulans* accessory gland cDNA library (Swanson et al., 2001).

Such intricate impacts of male products on female reproductive biology can be viewed as part of the general picture of sexual selection. Not surprisingly, similar functions of MAG

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products have been described in many other insects with a solitary life history. However, the social insects – and foremost the highly eusocial honey bee, *Apis mellifera* – deviate from the general pattern of insect reproductive biology and associated life histories (Heinze and Schrempf, 2008). The highly fertile female caste, the queen, has a much longer life span than the subfertile workers and, in the case of the honey bee, the queens may mate successively with 15 and more drones during one or few mating flights early in her adult life (Estoup et al., 1994). The male drones, in contrast, have a very short adult life span, as they either die during the in-flight copulation event or, the less lucky ones, later in season after being expelled from the colony by the workers. This difference in honey bee mating and reproductive biology in general, when compared to solitary insects, prompted our interest in the MAG products of honey bee drones.

After their emergence from brood cells, drones pass through an 8–10 day period of sexual maturation before they start flying to the congregation areas that are visited by virgin queens. During this period, their flight muscles are fully developed and they also complete the maturation of sperm in their reproductive system (Mindt, 1962). This maturation process appears to be controlled and synchronized by the endocrine system. Corpora allata activity and, consequently, the juvenile hormone titer gradually increases within the first days of adult life and exhibits a peak during this period of sexual maturation, promoting flight activity (Giray and Robinson, 1996; Tozetto et al., 1997). Concomitantly, the ecdysteroid titer drops and remains at basal levels during the rest of the drone's adult life (Colonello and Hartfelder, 2003).

The reproductive tract of a honey bee drone contains a pair of very large accessory glands (Snodgrass, 1956), also called mucus glands. During copulation, the drone everts an endophallus (Woyke, 2008) and transfers sperm together with a highly viscous mucus secretion. This secretion rapidly polymerizes when exposed to air. Considering that drones lack external genitalia and claspers, it may play an important role as a glue to maintain the drone attached to the queen until sperm transfer is completed. Subsequently, the endophallus ruptures and the mucus gland secretions form a mating plug that also contains cornual gland and bulbus secretions (Koeniger and Koeniger, 2000). Contrary to what one might expect, this mating sign is not a functional plug that prevents a queen from quickly re-mating with other drones in the congregation area. Rather, the following drone uses a special hair field on the genitalia to remove the mating sign (Koeniger and Koeniger, 2000).

Apart from speculations on serving as a glue during in-flight copulation, little is known about the products or functions of MAG secretions in bees. In fact, despite its role as a model organism for sociality in the Hymenoptera, it is not the honey bee that has taken a lead in this field but rather the bumble bee, *Bombus terrestris*. The mating plug that a bumble bee male inserts into the *bursa copulatrix* during sperm transfer is chemically relatively simple. It contains a large amount of a cyclic dipeptide in a mixture with four fatty acids that turn the secretion into a highly viscous mass (Baer et al., 2000). When testing the biological activity of the mating plug components, Baer et al. (2001) identified linoleic acid as the active compound that makes a female become unreceptive to other males.

In the honey bee *Apis mellifera*, we previously surveyed the proteins secreted into the lumen of the mucus glands. These glands secrete an enormous amount of protein during sexual maturation of the drones. The molecular mass of mucus proteins ranges from 25 to 174 kDa, but it is a set of only three proteins between 43 and 47.5 kDa that gradually becomes dominant in the protein spectrum (Colonello and Hartfelder, 2003). When comparing the mucus gland secretion of newly emerged drones to 8 day-old sexually mature drones, we observed a striking reduction in protein pattern complexity as the drones age. This increase in the amount of total protein, accompanied by a reduction in protein pattern complexity, was found to be contingent on the decreasing ecdysteroid titer. Injections of 20-hydroxyecdysone caused a delay in mucus gland maturation, impeding the protein content increase and conserving the complex protein pattern typical of newly

emerged drones (Colonello and Hartfelder, 2003).

Considering this background, the present study aimed at identifying genes that are differentially expressed in mucus glands of drones as they undergo sexual maturation in an attempt to characterize honey bee Acps and to attribute putative functions based on comparative genomic data. We employed a variant of a subtractive hybridization strategy, the Representational Difference Analysis (RDA), which selectively amplifies genes that are overexpressed in a specific biological context. The cDNA fragments amplified from RDA libraries of mucus glands from newly emerged and 8 day-old drones were sequenced and annotated against the honey bee genome sequence (The Honey Bee Genome Sequencing Consortium, 2006).

2. MATERIALS AND METHODS

2.1. RNA extraction from drone mucus glands

Frames containing drone brood were removed from Africanized honey bee hives kept at the Experimental Apiary of the University of São Paulo, Ribeirão Preto, Brazil. The frames were kept in an incubator at controlled temperature (34 °C) for the collection of drones emerging from the brood cells within a 24 h interval. These drones were either collected directly for mucus gland dissection, or they received a colored Opalith tag (Graze, Germany) and were returned to their respective hives to be collected eight days later.

The drones were anesthetized on ice and had their reproductive tracts removed. Mucus glands were dissected in sterile ringer solution, rinsed to remove secretion products, and then transferred to TRIzol reagent (Invitrogen) for RNA extraction. After extraction, aliquots of 2 μg total RNA were treated with DNase I (0.1 U, Promega) and RNA purity was checked by spectrophotometry.

2.2. Representational difference analysis (RDA)

The entire RDA protocol was run twice on mucus glands extracted from age-marked drones collected at different seasons. This way, four subtractive cDNA libraries were generated, two representing genes that are overexpressed in mucus glands of newly emerged (NE) drones and two for 8 day-old (8d) drones. The methodology employed was a cDNA Representational Difference Analysis (RDA) (Pastorian et al., 2000) adapted for applications in bees (Judice et al., 2006). For the NE libraries, cDNA of newly emerged drones served as the tester population that was hybridized against cDNA of the 8d library as the driver population. For the 8d libraries, the tester/driver combination was inverted. In brief, the protocol steps were as follows: 2 μg of each RNA sample was submitted to reverse transcription and long distance PCR (SMART PCR cDNA synthesis kit, Clontech). The double-stranded cDNAs (1 μg) were restriction digested with *Mbo*I (New England BioLabs), ligated to R-adapters (Tab. I), and PCR amplified following the protocol of Hubank and Schatz (2000) to generate the respective cDNA representations. Enrichment of differentially expressed transcripts was achieved in two successive rounds of PCR amplifications that employed different adapters (J and N, Tab. I) and sequential subtractive hybridizations of the tester to an excess of driver cDNA in ratios of 1:100 and then 1:800 (Hubank and Schatz, 2000). After each of these successive steps, the cDNAs were purified (GFX PCR purification kit, Amersham Biosciences).

This suppression subtractive hybridization strategy removes transcripts shared by the driver and tester populations and enriches the libraries for differentially expressed genes. The final difference products were ligated into pGEM-T Easy vector (Promega) for transformation of competent DH10β *E. coli* cells. Inserts from positive recombinants were sequenced using a BigDye Terminator Cycle Sequencing Ready Reaction protocol (Applied Biosystems) run on ABI-PRISM 377 or ABI-PRISM 310 (Applied Biosystems) automatic sequencers.

2.3. Bioinformatics protocols

In the E-Gene annotation pipeline (Durham et al., 2005), the sequencing reads were first filtered to detect and remove ribosomal RNAs and mitochondrial DNA, as well as bacterial and fungal DNA contaminants. Subsequently, vector sequences were trimmed using Crossmatch. Read quality was checked and reads were assembled by Phred-Phrap procedures. Valid ESTs were dynamically translated by BLASTX and compared to a non-redundant (nr) database (GenBank). Additional analyses against this database were performed using

Table I. RDA adapters and quantitative PCR primer sequences and their respective target genes.

RDA-adapters	Symbol	Sequence
	R12	5'- GATCTGCGGTGA -3'
	R24	5'- AGCACTCTCCAGCCTCTCACCGCA -3'
	J12	5'- GATCTGTTCATG -3'
	J24	5'- ACCGACGTCGACTATCCATGAACA -3'
	N12	5'- GATCTTCCCTCG -3'
	N24	5'- AGGCAACTGTGCTATCCGAGGGAA -3'
Target genes – real-time PCR		
acid phosphatase 1	Acph-1F	5'- TCACCAAGCATATGTTAGACG -3'
	Acph-1R	5'- CAGGAACGTGAGGATAATAAAG -3'
*CG*14516	CG14516F	5'- CAGCCATAAGGGAAGGAAAT -3'
	CG14516R	5'- GCGCGATTCTGAGATATGAG -3'
atlastin	atlF	5'- CCATTACGTGGATTTTCGTG -3'
	atlR	5'- GGATTGACTATCGAAAGCAC -3'
bitesize	btszF	5'- GGAGAGATACCGCGTTTGTG -3'
	btszR	5'- GACCAATCTGCTGCCTATGTG -3'
ypsilon schachtel	ypsF	5'- CAATCCGAGAAAGGCTGTAC -3'
	ypsR	5'- GTCGCTTATCGGCAGCATAA -3'
*CG*11791	CG11791F	5'- GACCAGGATACACTTGTACAC -3'
	CG11791R	5'- GATCCTTTAATTCAGGAGGAA -3'
14-3-3ε	14-3-3εF	5'- CCTGATAGAGCATGTCGTCT -3'
	14-3-3εR	5'- CCTTGCATGTCTGACGTCC -3'
stretchin-mlck	Strn-MlckF	5'- TCGGATCCAAAGAGGCATAAC -3'
	Strn-MlckR	5'- GATTAATAGCCTCGGGTCCTC -3'
kayak	kayF	5'- CGACAGATCCGCAGAGAAAG -3'
	kayR	5'- CCTGTTGCAGCTGTTGTATC -3'
polyprotein deformed wing virus	DWVF	5'- CGACATAGATGACTAGGCGC -3'
	DWVR	5'- CCTGGATTAGATTCCGATGT -3'
*GB*17584	GB17584F	5'- GAATACAGCGGTGAAGGAATC -3'
	GB17584R	5'- GTTTCTTGCCAGGTTGATGC -3'
*GB*18642	GB18642F	5'- CGAAGGCAAACGAGGAAGAG -3'
	GB18642R	5'- CGATGATGTGGCCGAGACTA -3'
*rp*49	RP49-F	5'-CGTCATATGTTGCCAACTGGT-3'
	RP49-R	5'-TTGAGCACGTTCAACAATGG-3'

the BLASTN and BLASTP tools. The ESTs representing orthologs of genes of known function in other organisms were clustered using Gene Ontology terms (Ashburner et al., 2000) attributed to their respective *Drosophila* orthologs.

BLASTX searches were then run against the Official Gene Set 1 (GLEAN3 predicted genes) of the honey bee genome database (www.hgsc.bcm.tmc.edu/projects/honeybee) (Elsik et al., 2006; The Honey Bee Genome Sequencing Consortium, 2006). For mapping the respective ESTs to the genomic scaffolds of the honey bee genome (Amel 3.0), we used ARTEMIS (version 8.0) as a platform (Rutherford et al., 2000). This allowed determining the exact position of EST reads within exons of predicted genes or within putative UTRs. In cases where ESTs localized outside of GLEAN3-predicted genes, the mapping by Artemis made it possible to check whether this region contained ORFs which, thus, may constitute elements of possibly novel protein coding genes.

2.4. Validation of differential gene expression by qRT-PCR

The developmental phase-biased expression of genes detected as RDA ESTs was validated for a set of twelve genes, eight from the NE library and four from the 8d library. Gene-specific quantitative PCR (qPCR) primers listed in Table I were designed using Gene Runner (version 3.05). For the qPCR analyses, first strand cDNAs were synthesized from RNA extracted from mucus glands of newly emerged and 8 day-old drones. We collected three independent samples (each composed of five pairs of mucus glands).

For specific comparisons of a viral capsid protein encoding gene, newly emerged drones were collected from a single source colony. These were either immediately dissected (15 individuals) for RNA extraction of abdominal carcasses and mucus glands, or they were marked and returned to the colony for collection and dissection when they were 8 days old. Worker bees were collected simultaneously from the same source colony and were also dissected for RNA extraction. In these preparations, care was taken to completely remove the intestinal tract. In the qPCR analyses for viral load, we used three independent samples for drones and four for workers, each sample consisting of tissue obtained from five individuals. All samples were analyzed in triplicate.

Extracted RNA (1 μg) was treated with 0.1 U DNaseI (Promega) at 37 °C for 40 min to remove possible DNA contaminants. First-strand cDNA synthesis was performed using a SuperScript II protocol (Invitrogen) at 42 °C for 50 min and 70 °C for 15 min. These cDNA products were used as templates in qPCR amplifications using a SYBR Green (Applied Biosystems) protocol in an ABI Prism 7500 detection system (Applied Biosystems). The amplification protocol for all genes was: 50 °C for 2 min, 95 °C for 10 min, 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. For each gene, we established standard curves by serial dilution and checked the dissociation curves for contaminants. The ribosomal gene *rp49* served as calibrator (Lourenço et al., 2008). Transcript abundance was calculated by the comparative C_T method (Pfaffl, 2001) and fold changes were expressed as $2^{-\Delta\Delta CT}$ values. Each sample was run in triplicate, and three independent cDNA samples were run per gene. Mean $2^{-\Delta\Delta CT}$ values between groups (newly emerged drones versus 8 day-old drones) were compared by t-test using Welch's correction for unequal variance; probabilities $P < 0.05$ were considered statistically significant.

3. RESULTS

3.1. General library characteristics

In the suppression subtractive hybridization approach to detect differential gene activity in mucus glands during sexual maturation of honey bee drones, we generated four RDA libraries comprising a total of 215 reads (Phred quality ≥ 20). When mapped to the honey bee genome sequence and compared to the database of GLEAN3-predicted genes, we found the following matches for the two libraries: In the mucus gland libraries from newly emerged drones, 93 ESTs mapped to coding sequences of 20 predicted genes and 26 reads matched to 10 genomic regions outside predicted genes. In contrast, in the RDA libraries generated from mucus gland RNA of 8 day-old drones, most of the 122 reads were attributable to overexpression of a viral gene and the remainder to only 4 honey bee genes. The honey bee EST sequences were deposited in GenBank EST database (accession numbers EX159740 to EX159841 for the NE libraries and EX159736 to EX159739 for the 8d libraries).

3.2. Representational difference analysis for mucus glands of newly emerged drones

ESTs sequenced from the libraries of differentially expressed transcripts in the mucus gland of newly emerged drones are listed in Tables II and III. The ESTs shown in Table II are those that could be mapped to exons or putative UTRs of predicted genes. The table lists the corresponding genomic region (oriented or unoriented linkage group), the identifier (GB number) of the Official Gene Set of the honey bee (Elsik et al., 2006; The Honey Bee Genome Sequencing Consortium, 2006), as well as the number of ESTs that clustered to each predicted gene. The BLASTP results report best matches to *Drosophila* or human orthologs and their respective similarity scores. Only matches to these genomic

Table II. ESTs sequenced from two Representational Difference Analysis (RDA) libraries of mucus gland mRNA of newly emerged drones. These ESTs were mapped to the honey bee genome and compared to non-redundant (nr) databases. Shown are the genomic location (Group in the Amel 3.0 assembly) and GLEAN3-predicted CDS (GB - gene number in Official Gene Set 1; The Honeybee Genome Sequencing Consortium, 2006), hit localization of ESTs (to CDS or putatve UTR) and the number of ESTs mapping to each site, the respective fly or human orthologs retrieved by BLASTP searches in GenBank, followed by the similarity index (e-value) and corresponding Gene Ontology terms obtained from Flybase (http://flybase.bio.indiana.edu/).

Group	GB	Localization	ESTs #	Ortholog (fly or human)	e-value	Biological process
GroupUn.1897	GB12546	CDS	28	acid phosphatase	2e-51	phosphate metabolism
Group10.14	GB17584	CDS	11	predicted hypothetical protein	5e-41	–
GroupUn.3	GB11965	CDS	06	myosin heavy chain	0.0	cytokinesis; mitosis; myofibril assembly; striated muscle contraction
Group7.11	GB14689	CDS	03	peptidase	0.0	proteolysis
GroupUn.1159	GB14853	CDS	02	atlastin	0.0	immune response
Group11.2	GB15345	CDS	02	CG2989	1e-171	cell-cell signaling; chitin and polysaccharide metabolism; signal transduction
Group9.1	GB13141	5'UTR - 53 bp and 214 bp	02	shaggy	1e-13	intracellular signaling cascade; Notch and Wnt receptor signaling pathway; negative regulation of smoothened signaling pathway; oogenesis; protein amino acid phosphorylation; regulation of proteolysis and transcription
Group11.26	GB13617	CDS	01	bitesize	3e-122	positive regulation of body size; positive regulation of cell size; synaptic vesicle transport
GroupUn.460	GB12819	CDS	01	CG14439	3e-176	transport
Group7.11	GB17753	CDS	01	aminopeptidase	5e-149	proteolysis
Group2.1	GB17562	CDS	01	CG8801	0.0	cell proliferation
Group16.5	GB11237	CDS	01	suppressor of variegation 3-9	0.0	DNA methylation; chromatin assembly or disassembly; chromatin silencing; establishment and/or maintenance of chromatin architecture; gene silencing; histone H3-K9 methylation; histone methylation; histone modification; oogenesis; translational initiation
Group16.10	GB18446	CDS	01	ypsilon schachtel	1e-22	oogenesis; regulation of transcription from RNA polymerase II promoter
Group13.4	GB19169	CDS	01	CG15105	6e-152	cell proliferation; nucleic acid metabolism; protein ubiquitination; regulation of transcription from RNA polymerase II promoter; transport
Group1.25	GB11183	3'UTR-253bp	01	basigin	2e-24	spermatid development
GroupUn.89	GB16541	CDS	01	CG11791	5e-70	–
Group2.5	GB18642	CDS	01	predicted hypothetical protein	0.0	–
GroupUn.447	GB12575	CDS	01	fau	7e-10	–
Group6.27	GB16247	CDS	01	KIAA1345	8e-24	–
Group8.18	GB17780	CDS	01	CG8483	5e-38	defense response

Table III. ESTs representing possible genes (ORFs identified by manual annotation in Artemis 8.0) detected as overexpressed in RDA libraries of mucus glands of newly emerged drones. Shown are the genomic location (Group in the Amel 3.0 assembly), the number of ESTs matching to each specific site, BLAST results from searches in GenBank, genomic span of contigs or singlets, and the respective GenBank accession numbers.

Group	ESTs #	BLASTN and BLASTX (EST × nr)	genomic span of contig or singlet (bp)	GenBank accession number
Group2.5	09	no match	464	EX159811 – EX159819
Group2.5	01	no match	190	EX159810
Group3.4	01	no match	136	EX159833
Group3.16	01	no match	457	EX159834
Group6.24	03	no match	348	EX159835 – EX159837
Group7.30	03	no match	128	EX159820 – EX159822
Group8.15	01	no match	48	EX159809
Group9.15	05	no match	600	EX159823 – EX159827
Group11.9	01	no match	72	EX159806
Group13.1	01	no match	153	EX159807
Group14.1	01	no match	166	EX159808
Group15.16	01	no match	159	EX159831
GroupUn.97	01	no match	2260	EX159832
GroupUn.416	01	no match	107	EX159828
GroupUn.5289	02	no match	218	EX159829 - EX159830

databases were listed because they best permitted to retrieve corresponding Gene Ontology information, when available.

The majority of ESTs in this library were identified as mapping to *acid phosphatase*-1 (28 ESTs), a gene encoding an allergen found in venom glands of female honey bees. The second in the list of predominant ESTs corresponded to the predicted *Apis mellifera* gene GB17584 (11 ESTs), for which no ortholog could be found in the nr database. Other genes represented by more than one EST were a predicted *myosin heavy chain* (6 ESTs), two peptidase genes (4 ESTs), *atlastin* (2 ESTs), *CG*2989 (2 ESTs) and *shaggy* (2 ESTs). In addition to the honey bee genes listed in Table II, we also sequenced 3 clones that contained the fragment of a *polyprotein deformed wing virus* gene encoding a protein of the viral capsid.

It is interesting to note that the predicted gene GB17584, even though represented here by 11 ESTs, has not been reported as an expressed gene in other honey bee EST or cDNA libraries, indicating that GB17584 may be a novel/unique honey bee gene that is primarily related to reproductive functions. In this respect, GB17584 differs from the other two genes in Table II for which we also could not obtain functional information (GB16541 and GB18642), since the latter has previously been reported as represented in a normalized bee brain cDNA library (Whitfield et al., 2002).

The results listed in Table III represent ESTs that did not fall within or close to GLEAN3-predicted genes but mapped to genomic regions that fell within ORFs that totally covered the respective ESTs. Since these ESTs showed frequency distributions to specific genomic regions similar to the ones that could be mapped to *in silico* predicted genes, we consider that they are valid expression tags that may serve for the identification of novel honey bee genes not predicted by the GLEAN3 algorithms. Since we did not retrieve any similar sequences when running BLASTN and BLASTX comparisons to the honey bee EST database, it is quite probable that these ESTs represent fragments of genes that are not only specifically expressed in mucus glands but are also genes that may

Table IV. ESTs sequenced from two Representational Difference Analysis (RDA) libraries of mucus gland mRNA of 8 day-old drones. These ESTs were mapped to the honey bee genome and compared to non-redundant (nr) databases. Shown are the genomic location (Group in the Amel 3.0 assembly) and GLEAN3-predicted CDS (GB - gene number in Official Gene Set 1; The Honey Bee Genome Sequencing Consortium, 2006), hit localization of ESTs (to CDS or putatve UTR) and the number of ESTs mapping to each site, the respective fly or human orthologs retrieved by BLASTP searches in GenBank, followed by the respective similarity index (e-value) and corresponding Gene Ontology terms obtained from Flybase (http://flybase.bio.indiana.edu/).

Group	GB	Localization	ESTs #	Ortholog (fly)	e-value	Biological process
Group7.26	GB15582	CDS	01	14-3-3ε	7e-129	DNA damage checkpoint; Ras protein signal transduction; imaginal disc development; maintenance of oocyte identity; mitotic checkpoint; nonassociative learning; oocyte microtubule cytoskeleton polarization; regulation of mitosis; response to external stimulus; response to radiation
Group7.15	GB16909	CDS	01	stretchin-mlck	0.0	chromosome segregation; cytokinesis; heart development; mesoderm development; mitosis; protein amino acid phosphorylation
Group5.11	GB12212	CDS	01	kayak	9e-18	JNK cascade; R3/R4 cell fate commitment; antimicrobial humoral response; central nervous system development; dorsal closure; establishment of planar polarity; follicle cell migration; head involution; imaginal disc fusion, thorax closure; midgut development, regulation of transcription, DNA-dependent, response to wounding, wound healing
Group14.4	GB18543	3'UTR -152 bp	01	hyaluronidase	0.0	–

have undergone rapid evolutionary change in their protein coding sequences.

3.3. Representational difference analysis for mucus glands of 8 days old drones

The majority (96.7%) of the 122 clones sequenced in this library were identified as representing a *polyprotein deformed wing virus* gene. This is a capsid protein of a single strand RNA inflavirus that is commonly found in honey bee colonies (Bailey, 1976). The four other ESTs transcripts were identified as representing fragments of *14-3-3ε*, S*tretchin-mlck*, *kayak*, and *hyaluronidase* genes. All these correspond to annotated genes in the honey bee genome (Tab. IV). Proteins of the 14-3-3 family are of interest because of their role in signal-transduction cascades that control cell cycle check points, activation of MAP kinases, apoptosis, and other effects on gene expression (Yaffe, 2002). Further genes of interest in this list are *kayak*, the *Drosophila c-fos* ortholog, and *hyaluronidase* which encodes an allergenic enzyme found in female honey bee venom glands.

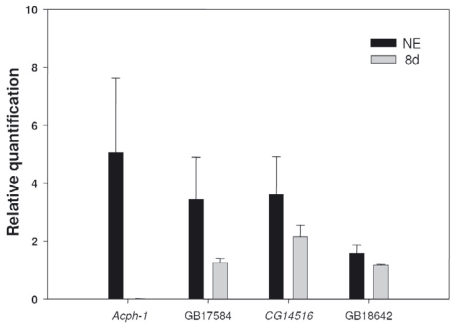

Figure 1. Relative quantification ($2^{-\Delta\Delta Ct}$) of *acid phosphatase*-1 (*Acph*-1), GB17584, *CG*14516, and GB18642 transcripts in mucus glands of newly emerged (NE) and 8-day-old (8d) drones. Shown are means ± S.E. of three independent samples, each run in triplicate.

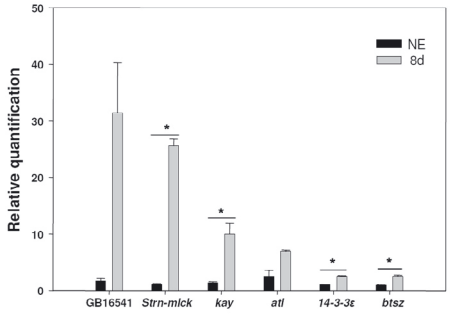

Figure 2

Figure 2. Relative quantification ($2^{-\Delta\Delta Ct}$) of GB16541, *Stretchin-mlck* (*Strn-mlck*), *kayak* (*kay*), *atlastin* (*atl*), 14-3-3ε, and *bitesize* (*btsz*) transcripts in mucus glands of newly emerged (NE) and 8-day-old (8d) drones. Statistically significant differences (t-test with Welch's correction, $P < 0.05$) are indicated by asterisks.

3.4. Validation of RDA results by qPCR for selected genes

The RDA protocol is a strategy to enrich and sequence differentially expressed genes, but like all results of high throughput approaches, the respective findings have to be validated by specific gene-expression analyses, such as quantitative real-time RT-PCR. To this end, we designed specific primers for the following representative set of genes from both library types: *acid phosphatase*-1, GB17584, *peptidase* (*CG*14516), GB18642, GB16541, *Stretchin-mlck, kayak*, *atlastin*, 14-3-3ε, *bitesize*, *ypsilon schachtel*, and *polyprotein deformed wing virus*. This set includes orthologs to genes of known function, as well as genes for which functions have not yet been defined.

The mean expression levels for *acid phosphatase*-1, GB17584, CG14516, and GB18642 appeared to be higher in mucus glands of newly emerged drones, yet these were not statistically different (*t*-test with Welch's correction, $P < 0.05$) from mucus glands of 8 day-old drones (Fig. 1). This is due to sample size and between-sample variation. In mucus glands of 8 day-old drones, the genes GB16541, *atlastin*, and *bitesize* of the NE RDA library showed higher mean expression levels, and these were statistically significant for *bitesize* (Fig. 2). Over-expression was also confirmed for S*tretchin-mlck*, *kayak,* and 14-3-3ε, which had been sequenced from the 8d RDA libraries.

3.5. Detection and quantification of deformed wing virus

The striking prevalence of ESTs corresponding to a capsid polyprotein of deformed wing virus (DWV) was confirmed by qPCR analyses of mucus glands dissected from newly emerged and 8 day-old drones (Fig. 3A). The high viral loads observed in these drone samples made us ask whether this is related to age (increasing viral infection as bees age) or to sex (drones *versus* workers). To answer this question, we compared *polyprotein deformed wing virus* transcript levels in abdominal carcasses of both sexes. The comparative analysis of transcript levels (Fig. 3B) revealed that worker bees have much lower DWV transcript levels than drones of the same age. The difference is actually around three orders of magnitude. Even though there appears to be a general tendency for an increase in viral load as drones undergo sexual maturation (Fig. 3A), we noted here (Fig. 3B) that

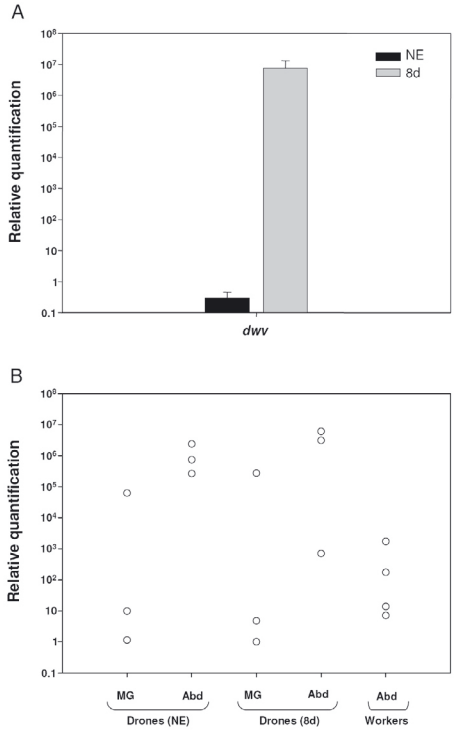

Figure 3. Relative quantification ($2^{-\Delta\Delta Ct}$) of *polyprotein deformed wing virus* transcripts in honey bee drones and workers. (A) Comparison of mucus glands of newly emerged (NE) and 8-day-old (8d) drones. Shown are means ± S.E. of three independent samples, each run in triplicate. (B) *Polyprotein deformed wing virus* transcript levels in mucus glands (MG) and abdominal carcasses (Abd) of newly emerged (NE) and 8-day-old drones in comparison to abdominal carcasses of workers of the same age. Shown are means of triplicate analyses of individual samples.

individual drones can have quite high viral loads already at emergence.

4. DISCUSSION

4.1. Differentially expressed genes in mucus glands of honey bee drones – functional considerations

For the *acid phosphatase* gene, we detected not only the highest number of ESTs mapping to a single predicted CDS, but also confirmed its over-expression in newly emerged drones by qPCR. The product of this gene has been described as an allergen found in venom glands of female honey bees (Barboni et al., 1987; Grunwald et al., 2006). Acid phosphatase activity was also detected in ovaries of larvae and in the intestine of worker pupae (Cruz-Landim et al., 2002). In addition, it seems to play a role in autophagic cell death, occurring in hypopharyngeal glands as honey bee workers age and start to forage (Costa and Cruz-Landim, 2002). Detection of *acid phosphatase* expression in mucus glands of adult drones is a novel finding, to which we cannot yet attribute a specific function.

Another interesting result was the identification of *hyaluronidase* transcripts in the 8 d mucus gland libraries. Like acid phosphatase, hyaluronidase is also an important venom allergen and hyaluronidase activity has previously been characterized in drone mucus glands (Allalouf et al., 1974). The current results are consistent with a positional homology between the hymenopteran female venom glands and the male mucus glands, both being accessory glands to the reproductive system. A large-scale comparative analysis of the accessory gland morphology of 54 bee species (Ferreira et al., 2004) supports gland homology, and it is especially interesting that in stingless bees the reduction of the sting apparatus in females is accompanied by the loss of accessory glands in the male sex. Further experiments are, however, needed to check to what extent this positional homology also translates to similarity in expressed gene products and their functions beyond the two genes for which we could find evidence.

Four genes with known functions were identified as over-expressed in mucus glands of 8-day-old drones. The protein Stretchin-MLCK is a novel member of the titin/myosin light chain kinase family (Champagne et al., 2000), and accordingly it may play a role in the structural organization of the actin/myosin cytoskeleton. The *kayak* gene encodes D-Fos, the *Drosophila* homolog of the mammalian proto-oncogene product c-Fos. Kayak is required in many developmental processes in *Drosophila*, such as endoderm induction (Riese et al.,

1997), dorsal closure during embryogenesis (Riesgo-Escovar and Hafen, 1997), organization of the follicular epithelium during oogenesis (Dequier et al., 2001), eye differentiation, and wing venation (Bohmann et al., 1994; Ciapponi et al., 2001). In honey bees, there is little information on c-Fos except for a study which reports immunodetection of Fos-like proteins in antennal lobe (Fonta et al., 1995), where it is thought to be involved in the integration and storage of odor signals.

Proteins belonging to the 14-3-3 family were the first signaling molecules to be identified as binding to phosphoserine/threonine residues and playing critical roles in cell cycle checkpoints, MAP kinase activation, apoptosis, and other programs of gene expression (Fu et al., 2000; van Hemert et al., 2001). The honey bee *bitesize* ortholog, sequenced from the NE RDA libraries but eventually revealed as overexpressed in 8 day-old drones, contains Pfam-predicted C2 domains which are known to act as protein interaction domains and to frequently mediate membrane association. They also regulate several aspects of intracellular traffic and vesicle fusion, as shown for the synaptotagmin-like proteins (Fukuda and Mikoshiba, 2001). In drone mucus glands, Bitesize may play a role in the very active secretion process observed during sexual maturation. Another protein possibly involved in intracellular traffic was also sequenced from the NE subtractive library and annotated as an ortholog of the fly gene *CG*14439. This gene shares similarity with MFS transporters, which are secondary carriers in the transport of low molecular mass solutes (Pao et al., 1998). *CG*14439 has a conserved sugar transporter domain and, thus, may participate in the carbohydrate shuttle from hemolymph into mucus glands, where they constitute a considerable part of the secretions (Gillott, 2003).

In addition to the ESTs for which we could identify orthologs in other organisms by BLASTX searches against the nr database, we also sequenced ESTs mapping to the CDS of two genes (GB17584 and GB18642) that were only predicted as hypothetical proteins. Their expression in mucus glands, which was confirmed by qPCR, makes it now possible to establish these as valid genes with possible reproductive functions.

4.2. RDA screens as tools to detect unique honey bee genes and to increase gene number

A question of general interest in genomics is gene number in a given species. The number of 10,157 predicted genes in the Official Gene Set (The Honey Bee Genome Sequencing Consortium, 2006) is certainly a conservative estimate reflecting the limited set of cDNA (Whitfield et al., 2002) and EST databases (Nunes et al., 2004) for the honey bee when compared to model organisms like *Drosophila*. It is, thus, not completely surprising that in the tissue-specific RDA libraries of drones, which are the neglected sex in honey bee research anyway, we found a considerable number of expression tags that did not match with any predicted gene. Three ESTs sequenced in our study mapped to intronic regions of GLEAN3-predicted genes and another 32 ESTs did not match any predicted gene in the Official Gene Set. The fact that they could all be unambiguously assigned to specific genome regions is strong evidence that they may represent novel genes that either encode proteins that were not predicted by the GLEAN3 algorithms (Elsik et al., 2006), due to low CDS conservation, or they could be non-coding RNAs, such as revealed in large amounts in tiling arrays of the human genome (Kapranov et al., 2007). However, since we could confirm the presence of ORFs for all of these no-match ESTs, it is quite probable that they indeed represent novel protein-coding genes specific to the honey bee.

This finding of a relatively large fraction of ESTs representing possibly novel, honey bee-specific genes expressed in the mucus glands of drones is in accordance with the results of the proteomics study by Collins et al. (2006) on seminal vesicle and semen of honey bee drones. In addition to identifying a large number of apparently conserved glycolysis-related proteins in drone seminal fluid that could play a role in sperm migration and storage in the spermatheca, they also noted that there are

only few orthologs to *Drosophila* Acps in the honey bee genome, suggesting fast evolutionary change in the secretion products of male accessory glands.

4.3. A viral gene overexpressed in mucus glands of sexually mature honey bee drones

In both RDA libraries generated from mucus gland mRNA of 8 day-old drones as tester, we retrieved an extraordinarily large number of clones corresponding to a *polyprotein deformed wing virus* gene. Its over-expression was validated by qPCR, showing that this mRNA is approximately 26 million times overrepresented in 8-day-old drones when compared to newly emerged drones. One explanation for the higher expression of the DWV gene in 8-day-old drones could be the infestation by varroa mites. A recent study has shown a positive correlation between viral RNA copy number and mite infestation levels for the respective bee hives (Yue and Genersch, 2005). Interestingly, honey bees can tolerate quite high DWV loads, showing little or no disease-related symptoms (Bowen-Walker et al., 1999; Gauthier et al., 2007), and this is probably the reason why we retrieved so many viral RNA positive clones in drone samples from apparently asymptomatic hives.

Possibly correlated with this finding is the over-expression of a honey bee *atlastin* ortholog, which is functionally annotated as involved in viral immune responses (GO:0005525; GO:0003924; GO:0006955). Whereas *atlastin* ESTs were sequenced from an NE subtractive library, the gene actually appears to be over-expressed in 8-day-old drones in the subsequent qPCR analysis. This gene, therefore, seems to be induced at low levels of viral infestation and continues to increase as viral titers rise.

The high levels of DWV transcripts in mucus glands of 8-day-old drones have interesting implications for DWV epidemiology. Viral loads in drone abdomens, and specifically in the accessory glands, were three orders of magnitude higher when compared to abdomens of workers of the same age. Furthermore, the detection of low levels of *polyprotein deformed wing virus* RNA in newly emerged drones that had not been in contact with workers suggests that *Varroa* mites may be the major route of transmission in this case, since these ectoparasitic mites preferentially infest drone brood shortly before the brood cells are capped (Fuchs, 1990). Epidemiologically, even more interesting could be the high viral load in mucus gland secretion, since this secretion is transmitted to virgin queens during copulation and, when founding new colonies after swarming, these colonies could gradually become DWV infected even when the worker population of the swarm itself is virus free. This idea is supported by a recent report on DWV localization in specific tissues in *Apis mellifera* (Fievet et al., 2006) and would classify DWV infection also as a sexually transmitted disease.

ACKNOWLEDGEMENTS

We are grateful to Luiz Roberto Aguiar for technical assistance in the apiary, to Carla Cristina Judice (Unicamp) for advice in the standardization of the RDA methodology, to Alexandre dos Santos Cristino for support in the bioinformatics analyses, to Anete Pedro Lourenço for helpful tips in qPCR analyses, and to Mendelson Mazucato for technical assistance in EST sequencing. This work was supported by FAPESP grants (99/00719-6, 02/06671-0 and 05/03926-5).

Expression génique différentielle dans les glandes à mucus des mâles d'abeilles (*Apis mellifera*) au cours de la maturation sexuelle.

***Apis mellifera* / glande accessoire / mâle / reproduction / virus de l'aile déformée**

Zusammenfassung – Differentielle Genexpression in Mucusdrüsen von Drohnen der Honigbienen *(Apis mellifera)* imVerlauf ihrer sexuellen Reifung. Bedingt durch ihre beschränkte Rolle im Lebenszyklus der Kolonien stellen die Männchen sozialer Hymenopteren das in Studien weitgehend vernachlässigte Geschlecht dar. Ihre Rolle beschränkt sich jedoch nicht nur auf ihren genetischen Beitrag, vielmehr beeinflussen sie auch die Reproduktionsbiologie der Königinnen durch die Übertragung der Sekrete ihrer akzessorischen Drüsen.

Bei Bienen ist wenig bekannt über die Funktion dieser Drüsenprodukte. Lediglich bei Hummeln wurde Linolensäure als Substanz identifiziert, die die Paarungswilligkeit von Königinnen reduziert. Die Identifizierung in Mucusdrüsen differentiell exprimierter Gene sollte dementsprechend unsere Kentnisse über mögliche Funktionen dieser Sekrete erweitern.

In einem *Representational Difference Analysis* (RDA) Ansatz sequenzierten wir ESTs aus subtrahierten Bibliotheken die aus Mucusdrüsen von frischgeschlüpften und 8-Tage alten Drohnen erstellt wurden. Aus den insgesamt 215 ESTs der Bibliothek von frischgeschlüften Drohnen zeigten 93 Übereinstimmungen mit 20 Honigbienengenen (Tab. II). Die restlichen Sequenzen zeigten zwar keine Übereinstimmung mit vorhergesagten Genen, konnten aber eindeutig 10 genomischen Regionen zugeordnet werden (Tab. III). Da alle ESTs innerhalb von ORFs lagen, repräsentieren diese möglicherweise neue Gene. Da diese Gene in einem für die Reproduktion wichtigen Organ gefunden wurden, könnten sie für künftige Studien zur Paarungsbiologie interessant sein. Im Gegensatz zu frischgeschlüpften Drohen fanden wir in Bibliotheken von 8-Tage alten Drohnen lediglich ESTs für vier Honigbienengene, während die meisten der 122 ESTs Übereinstimmung mit einem viralen Gen zeigten (Tab. IV).

Mittels quantitativer PCR verifizierten wir die differentielle Expression von 12 Genen in RNA-Proben von Mucusdüsen frischgeschlüpfter und 8 Tage alter Drohnen (Abb. 1 und 2). Von besonderem Interesse sollte die differentielle Expression von GB1754 and GB16541 sein, zwei Honigbienengenen, für die bishlang keine Funktion bekannt ist. Wir konnten auch die starke Überexpression des *polyprotein deformed wing virus* Gens in Mucusdrüsen 8-Tage alter Drohnen bestätigen (Abb. 3), was darauf hindeuten könnte, dass Könginnen über diesen Weg während der Paarung leicht mit DWV infiziert werden können.

Die differentielle Expression von *acid phosphatase-1* and *hyaluronidase* in Mucusdrüsen betrifft zwei Gene, die chakteristische Produkte der weiblichen Giftdrüsen kodieren. Dies könnte Evidenz dafür sein, dass diese mit dem Reproduktionstrakt in Verbindung stehenden Drüsen möglicherweise homologen Ursprungs sind. In weiterem Zusammenhang könnte die hier beschriebene differentielle Expression von Genen im Verlauf der sexuellen Reifung von Drohnen den Weg bereiten für künftige funktionelle genomische Untersuchungen, z.B. mittels RNAi-vermittelter Stillegung von Genfunktionen.

Männliche Anhangsdrüsen / *Representational Difference Analysis* / Honigbiene / Reproduktion / Verkrüppelte Flügel-Virus

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