Worker Honeybee Sterility: A Proteomic Analysis of Suppressed Ovary Activation

Dries Cardoen,^{*,†,‡} Ulrich R. Ernst,^{†,‡} Bart Boerjan,[†] Annelies Bogaerts,[†] Ellen Formesyn,[§] Dirk C. de Graaf,[§] Tom Wenseleers,[‡] Liliane Schoofs,[†] and Peter Verleyen[†]

[†]Research group of Functional Genomics and Proteomics, KU Leuven, Belgium

[‡]Laboratory of Entomology, KU Leuven, Belgium

[§]Laboratory of Zoophysiology, Ghent University, Belgium

S Supporting Information

ABSTRACT: Eusocial behavior is extensively studied in the honeybee, *Apis mellifera*, as it displays an extreme form of altruism. Honeybee workers are generally obligatory sterile in a bee colony headed by a queen, but the inhibition of ovary activation is lifted upon the absence of queen and larvae. Worker bees are then able to develop mature, viable eggs. The detailed repressive physiological mechanisms that are responsible for this remarkable phenomenon are as of yet largely unknown. Physiological studies today mainly focus on the transcriptome, while the proteome stays rather unexplored. Here, we present a quantitative 2-dimensional differential gel electrophoresis comparison between activated and inactivated worker ovaries and brains of reproductive and sterile worker bees, including a spot map of ovaries, containing 197 identified spots. Our findings suggest that suppression of ovary activation might involve a constant interplay between primordial oogenesis and subsequent degradation, which is probably mediated through steroid and neuropeptide hormone signaling. Additionally, the observation of higher viral protein loads in both the brains and ovaries of sterile workers is particularly noteworthy. This data set will be of great value for future research unraveling the physiological mechanisms underlying the altruistic sterility in honeybee workers.

KEYWORDS: *Apis mellifera*, ovary and brain proteome, worker sterility, deformed wing virus

■ INTRODUCTION

Honeybees display an extreme form of eusociality where one queen produces virtually all the offspring and tens of thousands female workers refrain from reproducing. The altruistic sterility of the female worker bees has fascinated scientists for decades.^{1,2} The main functional and morphological difference between the two different female casts in a honeybee society (queen vs workers) is the extent of ovarian development. In queens, both ovaries consist of 150–180 ovarioles, which are enlarged and produce up to 2000 eggs per day.³ The development of ovarioles in workers, on the other hand, is repressed during the late larval stages by means of apoptosis, due to the reduced amount of royal jelly in the diet.⁴ This results in ovaries with only 3–26 ovarioles.³ These ovaries stay inactivated throughout the adult life of a worker bee. Only when a colony becomes hopelessly queenless, for example, when a queen happens to die and the subsequent breeding of a replacement queen fails, up to 30% of the worker bees start to develop viable eggs.⁵ Since worker bees are unable to copulate, all such worker-laid eggs remain unfertilized and, due to the haplo-diploid sex determination, develop into males. The

regulatory network repressing the ovary activation in honeybee workers in the presence of the queen remains largely enigmatic.

One theory regarding the regulation of worker ovary development that is receiving increasing empirical support is the Reproductive Ground Plan Hypothesis (RGPH).^{6–10} This hypothesis states that the honeybee's reproductive division of labor is controlled by the same gene set that regulated the multiple switching between the reproducing/nonforaging stage (queen-like) and the nonreproducing/foraging stage (worker-like) in the hypothetically solitary ancestors. Amdam et al. expanded this model by suggesting that the same set of genes that determines whether a larva develops into a queen or a worker also regulates the division of labor within the foraging population.⁸ This idea was supported by the correlation between worker ovary development and pollen foraging in a line of bees selected for high-pollen hoarding. Today, this theory is discussed among evolutionary biologists.^{11–13} Worker reproductive capacity in the RGPH has been defined according to the number of ovarioles, and a correlation between the

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number of ovarioles and egg development has been reported for Africanized honeybees.¹⁴ However, we focus here on the presence or absence of developed oocytes inside the ovaries, independent of the number of ovarioles. Here we aim at identifying more proximate factors involved in the regulation of worker ovary activation in the honeybee.

Though the honeybee genome sequence was released in 2006¹⁵ and a number of microarray screenings have since been conducted to investigate, for example, task allocation and fertility,^{16–22} studies on this topic at the level of the proteome are still scarce.^{23,24} Using a 2-dimensional differential gel electrophoresis (2D-DIGE) approach, we compare proteome profiles of activated versus inactivated ovaries dissected from age-matched worker bees from hopelessly queenless colonies. In addition, 2D-DIGE proteome maps of brains from reproductive and sterile workers are compared. We hypothesized *a priori* that a small subset of yolk proteins would be up-regulated in activated ovaries with mature eggs and that several structural proteins would be over-represented in inactivated ovaries, considering that this rudimentary organ consists mainly of membranes. We feature a spot map of ovarian protein extracts displaying 197 identified spots, 119 of which are differential (61 up-regulated in activated ovaries, 58 up-regulated in rudimentary ovaries). Furthermore, comparing ovaries and brains from sterile and reproductive workers confirmed earlier findings that sterile worker bees have higher viral protein loads of the Deformed wing virus (DWV).²⁵ Remarkably, results from additional experiments on the RNA-level by means of quantitative real time PCR (qRT-PCR) do not correspond with the proteomics data.

MATERIAL AND METHODS

Sample Collection

Three honeybee colonies (ca. 10,000 bees), each headed by a singly mated queen (obtained from the DLR Fachzentrum für Bienen und Imkerei, Mayen, Germany), were kept at the apiary of the Laboratory of Zoophysiology, Ghent, following standard beekeeping methods. To initiate worker ovary activation, queens were removed and subsequent queen-rearing cells were annihilated. From the day of queen removal on, emerging bees were paint marked (Posca paint markers) and introduced in the colonies on a daily basis. For this purpose, a frame with sealed brood was placed in an incubator (Memmert Precision Incubator Model INE 700, 34 °C, high relative humidity), allowing us to collect the emerging bees every 24 h and to return them paint marked on the thorax (changing color every 24 h) to their original colony.²⁶ At 18 days of age, all marked bees were recaptured in the early morning and immediately anesthetized on ice.

Sample Preparation and 2D-DIGE

Ovary Samples. Ovary samples were dissected in insect saline buffer (150 mM NaCl, 10 mM KCl, 4 mM CaCl₂, 2 mM MgCl₂, 10 mM 2-[4-(2-hydroxyethyl)piperazin-1-yl]-ethanesulfonic acid) and categorized based on ovary activation. An ovary was scored as “fully activated” if at least one oocyte was larger than 1.1 mm and “completely inactivated” if the length of the largest oocyte was shorter than 0.3 mm (Figure 1). Bees with intermediate ovary activation were not included in this analysis. The dissected organs were immediately snap frozen in liquid nitrogen and stored at –80 °C until further analysis. Frozen bee ovaries were suspended in a lysis solution (7 M urea, 2 M thiourea, 4% CHAPS, 40 mM Tris, 1% DTT

Figure 1. Dissected worker ovaries. (A) Ovary, scored as completely activated (largest oocyte >1.1 mm, see arrowheads). (B) Ovary, scored as completely inactivated (largest oocyte <0.3 mm).

and Complete protease inhibitor, Roche). Each sample of inactivated ovaries consisted of at least 40 rudimentary ovaries in 80 μ L lysis solution. Each sample of activated ovaries consisted of 10 fully activated ovaries in 100 μ L of lysis solution. In total, 4 biological replicates, each comparing a sample of activated ovaries with a sample of rudimentary ovaries, were obtained for ovary 2D-DIGE comparison, originating from two colonies. In total, this experiment includes 40 and 160 activated and rudimentary ovaries respectively.

Brain Samples. Brain samples were dissected in insect saline buffer and contain the entire central nervous system from the head, including the optic lobes, ocelli and calyx. Each brain sample consisted of five brains, dissected from bees with either rudimentary or activated ovaries, that were suspended in 150 μ L of the lysis solution described earlier. The experiment included 12 replicates from three colonies (six replicates from colony one and three replicates from colony two and three). In total, this experiment includes 60 brains from reproductive bees and 60 brains of sterile bees.

Labeling. During all procedures, the samples were kept on ice or 4 °C as much as possible. The extracts were sonicated for 5 s (MSE Sanyo Soniprep 150). Then, the samples were centrifuged for 10 min at 15800 rcf and 4 °C. Supernatants were desalted by means of the PlusOne Mini Dialysis Kit (GE Healthcare), protein concentration was determined using the Bradford method²⁷ and the pH was measured (pH 315i, WTW and Biotrode pH 0–14, Hamilton). Fifty micrograms of protein for each ovary sample and 75 μ g for each brain sample were labeled with 250 pmol CyDye (GE Healthcare), following a balanced dye-swap protocol. The CyDye, dissolved in anhydrous dimethylformamide (Sigma-Aldrich), was added to the sample and incubated on ice in the dark for 45 min. The reaction was terminated by adding 1 μ L aqueous lysine solution (10 mM). Gel preparations and gel electrophoresis were performed as described.²⁸ After electrophoresis, the gels were scanned using an Ettan DIGE Imager (GE Healthcare) according to manufacturer's instructions. The images of the analytical gels were imported and processed in the Decyder 7.0 software (GE Healthcare). Subsequently, raw spot volumes for the Cy3 and Cy5 channels were exported and analyzed using the Bioconductor package *limma*.²⁹ To perform the statistical analysis on the spots that consistently appear in all gels, we filtered the spot list for spots that were detected in at least 75% of the gels. The Cy3 and Cy5 channels were normalized within and between gels using loess and quantile normalization respectively. Finally, differential expression was measured according to the Log₂ spot volume ratios and its statistical significance was assessed using empirical Bayes moderated *t*-

tests carried out in *limma*.^{25,30–33} In this analysis, colony was included as a random blocking variable and *p*-values were corrected using Benjamini-Hochberg procedures³⁴ to control the false discovery rate (FDR). All spots with an FDR-adjusted *p*-value smaller than 0.05 were considered as differentially expressed and were used for further analysis. All *p*-values mentioned are FDR-adjusted. A full description of the 2D-DIGE protocol used here is available at the Minimum Information About a Proteomics Experiment Gel Database (MIAPEGelDB: Bee_Ovary_prot; http://miapegelddb.expasy.org/experiment/91/gel/208/as_html/).

Protein Identification

Preparative gels were run with protein extracts from activated and inactivated ovaries as well as brain extracts. Plus One Bind-Silane and two reference markers (GE Healthcare) were added to the gels, according to manufacturer's guidelines. Each preparative gel contained 400–600 μ g of protein. After 2D electrophoresis, the gels were stained with Deep Purple Total Protein Stain (GE Healthcare) and imaging was performed using the Ettan DIGE Imager. The images were imported in the Decyder 7.0 software and matched against the analytical gels. Pick lists contained the differentially expressed spots (for both the ovary and brain experiment) and consistently appearing spots (only for the ovary experiment). The spots were picked with the Ettan Spot Picker (GE Healthcare) and stored in Milli-Q water at -20°C until tryptic digestion. Gel pieces were dehydrated three times with 50 μ L of acetonitrile, after which they were rehydrated with 50 μ L of 100 mM of ammonium bicarbonate for 10 min. Gel pieces were subsequently dehydrated in 50 μ L of acetonitrile for 10 min. The last two steps were repeated twice. The enzymatic digestion was initiated by adding 25 μ L of digestion buffer (50 mM ammonium bicarbonate and 5 mM CaCl_2) containing 6 ng/ μ L trypsin (Promega). After 45 min incubation on ice, the enzymatic digestion was continued overnight at 37°C . The resulting peptides were extracted once with 80 μ L of 50 mM ammonium bicarbonate (30 min.) and two times with 80 μ L of 50% acetonitrile and 5% formic acid (each time 30 min). Supernatants of the three extractions were pooled together and dried in a vacuum centrifuge.

The dry extracts were desalted using Ziptip C_{18} (Millipore) and spotted on a MALDI Target ground steel plate. The samples were mixed in a 2:1 (v:v) ratio with α -cyano-4-hydroxycinnamic acid matrix (saturated solution in 50% acetonitrile, 0.5% formic acid). The spots in the ovary experiment were measured with a MALDI-TOF device (Reflex IV, Bruker). Settings of the mass spectrometer were adopted from Vierstraete et al.³⁵ with minor adjustments. External calibration was done with Peptide calibration standard (1000–4000 Da, Bruker Daltonics). Laser power was optimized for best resolution and signal-to-noise ratio. Spots with no or an inadequate identification were reanalyzed using an Ultraflex II mass spectrometer (Bruker). Postacquisition processing was carried out in Flex Analysis (Bruker Daltonics) and consisted of baseline subtraction (TopHat) and smoothing (Savitzky Golay). The peak detection in Flex Analysis was carried out using the SNAP algorithm (S/N ratio, 3; max. number of spots, 150; quality factor threshold, 30). The proteins were identified through peptide mass fingerprinting (PMF) using Mascot,³⁶ against the protein database of the *Apis mellifera* official gene set prerelease 2 (containing 11062 sequences), (http://genomes.arc.georgetown.edu/Amel_pre_release2_OGS_pep.fa). Carba-

midomethylation was set as fixed modification, oxidation (M) as variable modification and up to one missed cleavage was allowed. The mass tolerance was set at 0.2 Da as a default value. In addition, every spectrum was also searched against the publicly available NCBI database as a control for potential contamination with e.g. human keratin. Virus proteins from the brains were identified by means of MALDI-TOF/TOF mass spectrometry (UltraFlex II, Bruker Daltonics) and Mascot MS/MS Ion searches against the *Apis mellifera* official gene set release 1 (containing 9939 sequences), available at Beebase, and NCBI as a control. Both the peptide mass tolerance and fragment mass tolerance were set at 0.2 Da; carbamidomethylation (C) was a fixed modification and oxidation (M) was set as variable modification.

Functional Gene Ontology Enrichment Analysis

In the comparison of the proteome of fully activated and inactivated honeybee worker ovaries, a sufficient number of differentially expressed proteins (119) was obtained to be able to carry out a functional gene ontology (GO) enrichment analysis. We analyzed the differentially expressed ovarian gene set *in silico*. Using the Bioconductor *TopGO* package, in particular the modified hypergeometric test-based method “elim”,³⁷ we determined which biological processes are significantly enriched in the sets of differential proteins from activated and rudimentary ovaries. In case multiple spots contain the same protein, only the spot with the largest log ratio was used in the analysis. Gene ontology annotation information was collected by making use of various databases and resources, including Blast2Go, Pfam, Flybase, DAVID and Uniprot.³⁸ This resulted in the successful annotation of 154 out of 162 identified proteins with Biological Process Gene Ontology (GO) terms. In all cases, enrichment was tested relative to the remainder of the proteins identified in this study. Enrichment relative to the rest of the genome was not tested given the inherent biases and limitations of the 2-DE technique, for example, the fact that highly hydrophobic proteins, extremely heavy proteins and small peptides are not detected.³⁹

RNA Analysis of Deformed Wing Virus

Additional samples were collected from two colonies, each headed by a naturally inseminated, multiple-mated queen. Queen and open brood from both colonies were removed at the same time. Following standard procedures, broodframes with emerging brood from the experimental colonies were kept in an incubator at 34°C . Bees were paint marked, according to their age, within 24 h after emergence and introduced into their colony of origin. Marked bees of 18-days old were recaptured and the ovary activation was scored. As RNA extraction from rudimentary ovaries did not deliver RNA of sufficient quality, even when employing an RNA extraction kit suited for small tissues (RNeasy Micro, Qiagen), we opted to sample complete abdomens and dissected brains. RNA was extracted by means of the RNeasy Lipid Tissue kit (Qiagen) following manufacturers' guidelines. RNA concentration and quality was determined using Nanodrop ND-1000 (Nanodrop Technologies). Seven replicates of two colonies, resulting in 14 biological replicates were included both for entire abdomen samples and for dissected brain samples. Both DWV and the reference gene (GB10903) were detected by a one-step qRT-PCR protocol with Taqman probes (DWV-Fw: GTTAATCAGCGCT-TAGTGGAGGAA; DWV-Rev: CCGCACCTACGCGATGTAA; DWV-probe: AGGAGCGTACACTATGGT; GB10903-Fw: CAGTAAATTAAAGAGAACTGGCGTAAA; GB10903-

Annotation	Spot Nr	BeeBase Nr	Ratio (infertile/fertile)	Adjusted p- value	Gel Nr			
					1	2	3	4
similar to Lipid storage droplet-2 CG9057-PA	1957	GB14434	-12,82	0,0012				
vitellogenin	293	GB13999	-11,35	0,0012				
similar to Hsp70/Hsp90 organizing protein homolog	965	GB19425	-7,44	0,0012				
vitellogenin	298	GB13999	-7,05	0,0019				
FK506-binding protein FKBP59	1406	GB13770	-6,14	0,0012				
vitellogenin	1697	GB13999	-5,64	0,0038				
arginine kinase	1605	GB10973	-5,15	0,0043				
similar to squid CG16901-PC, isoform C	1808	GB15796	-5,03	0,0012				
similar to squid CG16901-PC, isoform C	1780	GB15796	-4,98	0,0011				
superoxide dismutase 1	2635	GB10133	-4,76	0,0025				
similar to 60 kDa heat shock protein	1008	GB18969	-4,67	0,0025				
heat shock protein 90	2736	GB14758	-4,63	0,0055				
calponin-like protein Chd64	2507	GB12375	-4,47	0,0014				
glutathione peroxidase-like 1	2542	GB14138	-4,33	0,0012				
arginine kinase	1649	GB10973	-4,01	0,0085				
similar to Cofilin/actin-depolymerizing factor	2614	GB18917	-3,77	0,0039				
thioredoxin peroxidase 1	2453	GB19380	-3,71	0,0017				
similar to Cofilin/actin-depolymerizing factor	2644	GB18917	-3,25	0,0078				
ATP synthase, H ⁺ transporting	2734	GB14791	-3,05	0,0034				
similar to CG6459-PA	2730	GB10107	-3,03	0,0037				
vacuolar H ⁺ -ATPase 26kD E subunit	2080	GB12913	-2,96	0,0032				
similar to Aldehyde dehydrogenase	1112	GB12741	-2,87	0,0025				
14-3-3 protein zeta	2211	GB12951	-2,80	0,0015				
similar to Vacuolar H ⁺ -ATPase 55kD B subunit	1048	GB19171	-2,59	0,0046				
phosphoglycerate mutase	2160	GB15052	-2,52	0,0037				
ATP-dependent RNA helicase me31b ortholog	1321	GB16998	-2,35	0,0254				
heat shock protein 70Cb ortholog	406	GB10836	-2,33	0,0031				
heat shock protein cognate 4	779	GB14852	-2,29	0,0162				
similar to Glycoprotein 93 CG5520-PA	480	GB12703	-2,14	0,0038				
ERp60 protein	1042	GB10238	-2,13	0,0174				
similar to CG32068-PA	2412	GB19130	-2,10	0,0041				
heat shock protein cognate 5	764	GB19860	-2,09	0,0048				
26S proteasome non-ATPase regulatory subunit 13	1572	GB16614	-2,05	0,0035				
similar to CG7033-PA, isoform A	1018	GB10699	-2,02	0,0143				
heat shock protein cognate 5	774	GB19860	-2,02	0,0058				
Tat-binding protein-1	1333	GB14678	-2,01	0,0116				
heat shock 70 kDa protein cognate 3	743	GB15016	-2,00	0,0452				
T-complex protein 1 subunit eta	1033	GB10561	-1,98	0,0044				
heat shock 70 kDa protein cognate 3	742	GB15016	-1,97	0,0043				
ERp60 protein	1029	GB10238	-1,88	0,0097				
similar to CG14207-PB, isoform B	2297	GB15718	-1,88	0,0246				
heat shock protein 70Cb ortholog	395	GB10836	-1,85	0,0085				
t-complex chaperonin 5	1031	GB10587	-1,83	0,0065				
similar to Tropomyosin 1 CG4898-PD, isoform D	2064	GB17608	-1,83	0,0221				
similar to Proteasome p44.5 subunit CG10149-PB	1431	GB18440	-1,81	0,0055				
soluble NSF attachment protein	1982	GB15709	-1,77	0,0089				
similar to Vacuolar ATP synthase catalytic subunit A	772	GB10989	-1,76	0,0238				
puromycin sensitive aminopeptidase	540	GB13772	-1,73	0,0459				
6-phosphogluconate dehydrogenase, decarboxylating	1304	GB12949	-1,73	0,0322				
similar to Dipeptidyl aminopeptidase III CG7415-PC	710	GB19373	-1,71	0,0167				
ALG-2 interacting protein X	543	GB11744	-1,67	0,0083				
GDP dissociation inhibitor	1222	GB13730	-1,62	0,0242				
heat shock protein cognate 4	758	GB14852	-1,60	0,0393				
Rpn5 protein	1282	GB11260	-1,57	0,0393				
ATP synthase subunit beta, mitochondrial	1263	GB13596	-1,56	0,0486				
similar to CG3011-PA	1285	GB14485	-1,55	0,0153				
similar to CG8036-PB, isoform B	904	GB15619	-1,54	0,0162				
thioredoxin peroxidase 3	2417	GB10972	-1,53	0,0179				
similar to Sterol carrier protein X-related thiolase	1090	GB10657	-1,49	0,0224				

Figure 2. continued

Figure 2. continued

Figure 2. continued

Figure 2. Overview of all differentially expressed proteins, displaying the annotation, spot number (according to Figure S1 and Table S1, Supporting Information) together with the BeeBase number (GB identifier) and the Log₂ ratio (infertile/fertile) and matching adjusted *p*-value. For each protein, the Log₂ ratio for each gel is presented in a heat map.

Table 1. Summary of the GO-Enrichment Analysis using TopGO, in the Differential Proteomics Experiment of Ovaries^a

nonreproductive workers					reproductive workers				
GO biological process term	GO nr.	NS	FE	<i>p</i> -value	GO biological process term	GO nr.	NS	FE	<i>p</i> -value
Metabolism					Metabolism				
Carbohydrate catabolic process	0016052	9	1.91	0.0132	ATP synthesis coupled proton transport	0015986	5	2.84	0.004
Organic acid metabolic process	0006082	11	1.72	0.0169					
Glycolysis	0006096	6	2.23	0.0172					
Cellular lipid metabolic process	0044255	6	2.23	0.0172					
Alcohol catabolic process	00416164	8	1.83	0.0289					
Pyruvate metabolic process	0006090	4	2.38	0.0433					
Fatty acid metabolic process	0006631	4	2.38	0.0433					
Egg Development					Stress and Protein Folding				
Neg. regulation of cell. component organization	0051129	5	2.98	0.0036	Protein folding	0006457	13	2.17	0.0003
Neg. regulation of organelle organization	0010639	4	2.99	0.0116	Protein complex assembly	0006461	5	2.84	0.0046
Neg. regulation of cell cycle	0045786	3	2.97	0.0362	Response to stress	0006950	13	3.30	0.0364
Neg. regulation of nuclear division	0051784	3	2.97	0.0362	Response to heat	0009408	3	2.86	0.0416
Other					Egg Development				
Locomotor behavior	0007626	3	2.97	0.036	Vesicle mediated transport	0016192	6	2.44	0.0076
					Cell. macromolecular complex assembly	0034622	4	2.84	0.014
					Pathways				
					Intracellular signaling cascade	0007242	3	2.86	0.0416

^aThe GO biological process terms, which are enriched in the differentially expressed protein set of either reproductive or sterile worker bees are depicted, together with their GO-numbers (NS: the number of differentially expressed proteins in the set, with the annotated GO-term; FE: fold enrichment). Full results are available in Tables S2 and S3 (Supporting Information).

Rev: GCATCAAATATTGTCCCTTAAACG; GB10903-probe: AAAGGTATTGATAACAGAGTTC). Reaction volumes consisted of 5 μ L of TaqMan Fast Virus 1-Step Master Mix (Applied Biosystems), 12 μ L Milli-Q water and 1 μ L of the Custom TaqMan Gene Expression Assays (20 $\times$ concentration, Applied Biosystems) containing previously mentioned primers and probes for each target specifically. The reaction volume was completed with 2 μ L of total RNA, resulting in a total volume of 20 μ L. Real time PCR was run by a StepOne Plus (Applied Biosystems) starting the reverse transcriptase step at 50 $^{\circ}$ C for 5 min, followed by 20 s at 95 $^{\circ}$ C to degrade the reverse transcriptase. Immediately after, the real time cycling started for 15 s at 95 $^{\circ}$ C and 1 min at 60 $^{\circ}$ C and was repeated 50 times. Ct values were obtained through the StepOne Real-Time System Software (Applied Biosystems). Relative transcript abundances were assessed by means of the delta delta Ct method.⁴⁰

RESULTS

Ovary Proteome

The quantitative comparison between fully activated and rudimentary honeybee worker ovaries resulted in an initial set of 613 differentially expressed features out of 1666 detected spots (277 higher in activated ovaries and 336 higher in rudimentary ovaries). In total, 119 differential spots could be identified, of which 58 were up-regulated in ovaries from sterile workers and 61 in activated ovaries. A further 78 additional random spots were identified to complete the spot map, resulting in a total of 223 identified proteins from 197 spots

(Figures 2 and S1, Table S1, Supporting Information, WORLD 2-Dimensional Differential Gel Electrophoresis Constellation at Expert Protein Analysis System, Expasy WORLD-2DPAGE experiment 29; <http://world-2dpagexpasy.org/repository/database=0029>). Gene Ontology (GO) enrichment analysis results are summarized in Table 1; complete data are available in Tables S2 and S3, Supporting Information. Surprisingly, one of the spots with the highest up-regulation in rudimentary ovaries (ratio = 3.8; *p* = 0.006) is the polyprotein of the Kakugo-virus (KV).

Brain Proteome

Whereas 1514 spots are detected in 75% of the gels, only two spots are significantly differential. The first is up-regulated by 4-fold in sterile workers (*p*_{adj} = 0.004), but could not be identified. The second spot (2517), identified as the polyprotein from the DWV, is enriched nearly 9-fold in the sterile workers (*p*_{adj} = 0.045). All details are summarized in Table S4 (Supporting Information). We identified tryptic peptide fragments from the VP3 part of the polyprotein.⁴¹ We detected DWV protein in brains of sterile workers originating from two out of three investigated colonies; in the third colony no DWV was detected at all (Figure 5). When omitting the three replicates from the colony in which no viral proteins were detected, the nine replicates of the two infected colonies, revealed an 18-fold increase of virus protein in sterile workers (*p*_{adj} < 0.001), which reflects an on/off situation as no signal

Figure 3. Overview of all relative abundances of DWV transcript in brain and abdomen. Samples originate from sterile (S) and reproductive (R) bees, collected from two colonies (C1 and C2). Relative abundance is displayed for each sample.

above the background was detected in reproductive workers (Figure 5).

Functional Enrichment Analysis

The enrichment analysis on the set of differential proteins up-regulated in activated ovaries results in significant enrichment of 18 GO terms (Tables 1 and S2, Supporting Information). Enriched terms deal with protein folding, response to heat and stress and subcellular transportation, endocytosis and phagocytosis. The enrichment analysis on proteins up-regulated in rudimentary ovaries results in 43 significantly enriched GO terms (Tables 1 and S3, Supporting Information) involving GO-terms dealing with the cell cycle and cell division, and metabolism of sugar, pyruvate and alcohol.

RNA Analysis of Deformed Wing Virus

We detect the DWV virus in nearly every bee (Figure 3). A small number of bees contain 10- to 100-fold more viral RNA than all other samples; however there is no correlation between the samples from reproductive and sterile bees. In particular, two abdomens in colony one and one abdomen in colony three contain remarkably high viral loads; all three were sterile bees. As for brains, five samples (one from a sterile bee, four from reproductive bees) are found to contain more virus than all 23 others. Three of these samples originate from colony one and two from colony two (Figure 3).

DISCUSSION

Despite an overall resemblance of the 2-dimensional gel images of active and rudimentary ovaries (Figure 4), there are pronounced differences which were quantitatively analyzed by means of a 2D-DIGE experiment. We expected a subset of yolk-proteins to be nearly uniquely expressed in activated ovaries and could observe that the major yolk protein, vitellogenin (Vg), is largely up-regulated (e.g., spot nrs. 293 and 298; up to 11-fold; $p = 0.0012$). We also expected that

Figure 4. Representative 2D-DIGE image acquired with Ettan DIGE Imager, displaying the inactivated worker ovary proteome (green) and activated worker ovary proteome (red). Identified spots are indicated with corresponding spot number in Figure S1 (Supporting Information).

structural proteins would be relatively enriched in rudimentary ovaries and we found that tubulin and actin-87E, for instance, here contained in several differential spots, have ratios higher than 2 (e.g., spot nr. 1522, 1101; $p < 0.01$). Remarkably, 47 of the identified proteins have previously been demonstrated to be part of the spermathecal fluid of honeybee queens.⁴² Spermathecae in worker bees are rudimentary, that is, they are barely visible while dissecting. Nevertheless, 31 of the 47 spermathecal proteins are differentially expressed, 15 of which are up-regulated in rudimentary ovaries and 16 are up-regulated in activated ovaries (Table S1, Supporting Information).

Consequently the differential expression of these proteins is probably not due to possible contamination by the rudimentary spermathecae in the ovary samples.

When we compare the present ovary proteomics data with published data obtained by microarray studies on worker ovary activation (the work of G. Thompson and C. Grozinger^{20–22} and our own work³⁸), we found no significant overlap. However, microarray experiments studying the effect of brood pheromone¹⁸ and queen mandibular pheromone (QMP)¹⁹ did reveal common differential genes depicted in Table S1 (Supporting Information). Examples are highlighted in the following paragraphs.

Functional Enrichment Analysis

The enrichment analysis (Tables 1, S2 and S3, Supporting Information) shows that proteins linked to the metabolism of several substrates (carbohydrate, alcohol, lipid, pyruvate, fatty acid, etc.) are up-regulated in rudimentary ovaries. This more than 2-fold enrichment (FE) points to an enhanced metabolism. As expected for inactive ovaries, the cell cycle and cell organization might be predicted to be inhibited, based on the enriched GO-terms in the ovaries of sterile bees (FE of nearly 3) that are associated with negative regulation of cell cycle, nuclear division, organelle organization etc. This conclusion, however, is based on the differential expression of only 3 identified proteins—two isomorphs of tubulin $\alpha 1$ (GB10009 and GB10514) and aldolase (GB19460)—which are also known to have various other functions and their main function is not solely the regulation of the cell cycle or nuclear division. Moreover, the up-regulation in inactivated ovaries of the proteins *vibrator* (GB10590; ratio = 1.6; $p = 0.016$)^{43,44} and *hu li tai shao* (GB 15113; ratio = 1.5; $p = 0.02$)^{45,46} points to an ongoing oogenic process. This finding argues against the straightforward interpretation of the repression of ovary activation as a one-way process. Suppression of ovary activation might involve a constant interplay between primordial oogenesis and subsequent degradation.⁴⁷ Indeed, this may explain the enduring potency of honeybee workers to activate their ovaries. In further support of this idea is that protein degradation-related proteins, such as Cathepsin D (GB16903; ratio = 1.84; $p = 0.02$), proteasome subunit alpha type 1 (GB11284; ratio = 1.76; $p = 0.01$) and the proteasome 25kD subunit (GB10390; ratio = 1.84; $p = 0.005$) are also up-regulated in the ovaries of sterile workers. A continuous primordial oogenesis and subsequent degradation could also account for the enrichment of metabolism-linked GO-terms described above. Although we did not identify proteins that directly point toward the suppression of ovary activation, the receptor of activated protein kinase 1 (GB11076; ratio = 2.1; $p = 0.003$), which is up-regulated in suppressed ovaries, might be part of a key regulating network in the activation process. Protein kinases are well-known components of signaling pathways which are often activated by the binding of a ligand to a G-protein coupled receptor. Unfortunately, G-protein coupled receptors and their ligands (mostly peptides) are not detectable using a 2D-DIGE approach,³⁹ but we did find that the β -subunit (GB15307; ratio = 3.7; $p = 0.001$) of one G-protein is up-regulated in inactivated ovaries. In addition, the protein annotated as thioredoxin peroxidase (GB19380, ratio = -3.7 , p -value = 0.002) and known to inhibit apoptosis⁴⁸ is up-regulated in activated ovaries. Inhibition of apoptosis is mandatory to let the oocytes develop into viable, fully developed eggs.

Proteins annotated with GO-terms related to oogenesis would be expected to be enriched in activated ovaries. Yet, we could not find specifically enriched GO-terms that are associated with meiosis or egg development as such. Nevertheless, one of the significantly enriched GO-terms in activated ovaries is vesicle mediated transport (GO:0016192; FE: 2.4; Tab. 1). Through endocytosis, the developing eggs take up large amounts of maternal products, such as RNA and vitellogenin. The ATP needed for this active transport and uptake, is probably produced within the ovaries or eggs as the term ‘ATP synthesis coupled proton transport’ is enriched as well (GO:0015986; FE: 2.8; Table 1).

Putative Key Regulating Proteins

Twelve out of the 66 up-regulated proteins in activated ovaries are chaperones or heat shock protein-linked proteins. These heat shock-proteins cause the enrichment of the GO-terms “response to stress” (GO:0006950; FE: 3.3) and “response to heat” (GO:0009408; FE: 2.9) (Table 1). In mice, heat shock proteins (hsp) and chaperones are major constituents of oocytes.⁴⁹ Moreover, it has been reported that hsp 70 and hsp 90 are present on the oolemma.⁵⁰ An explanation put forward is that hsps act as chaperones in the incorporation of proteins in the *zona pellucida* during its formation.⁵¹ However, no evidence for this hypothesis has been presented yet. Interestingly, in vertebrates, hsp 70 and hsp 90 are also known to be part of the steroid receptor complex.⁵² An essential part of this complex is one or more proteins containing tetratricopeptide repeats, such as FKBP52.⁵³ We found the *Apis mellifera* homologue, FKBP59 (GB13770; ratio = -6.1 ; $p = 0.001$), up-regulated in activated ovaries in this study (Table S1, Supporting Information) and it has also been detected in a microarray study showing a down-regulation in worker brains upon QMP-treatment.¹⁹ In honeybees, the queen signals her presence to the worker bee population by means of pheromones, including QMP. In the presence of QMP, the worker caste remains sterile. FKBP59 is a binding protein of FK506, which is an agonist for TOR (Target of rapamycin) and necessary for the development of a larva into a queen.⁵⁴ FKBP46, which is part of the *ultraspiracle-ecdysone receptor* complex, was not identified.⁵⁵ The hsp 90 organizing protein (GB19425; ratio = -7.4 ; $p = 0.001$; Table S1, Supporting Information) is known to be part of the 20-hydroxyecdysone (20E) receptor complex⁵⁶ and is more than 7 times up-regulated in activated ovaries, and the transcripts of this gene are down-regulated in worker brains upon QMP-treatment.¹⁹ In addition, spot 1090 is significantly up-regulated in activated ovaries, and contains a protein identified as “similar to Sterol carrier protein X-related thiolase CG17320-PA” (ratio = -1.5 , p -value = 0.02). One of the GO-terms of this protein is the binding of sterol. Although we did not identify a steroid receptor in this experiment, the identification of differential occurrence of heat shock proteins, proteins containing tetratricopeptide repeats and a potential carrier of steroid hormones could suggest a role for steroid signaling in oogenesis in worker bees, which so far has not been demonstrated.^{57,58} This suggestion is in agreement with a transcriptomic study, that reported that Ecdysone-induced gene 74 and the Broad complex are significantly up-regulated in the whole body of reproductive worker bees, when compared to sterile bees.³⁸ These results shed a new light on the role of ecdysteroids on honeybee worker reproduction, which has been contested in the past.^{57–59}

Figure 5. Representative 2D-DIGE image of the brain proteome, displaying samples of sterile worker bees in red and reproductive worker bees in green. The 3D-images clearly demonstrate the presence of the DWV protein in the sterile bees of colonies 1 and 2 (upper graphs in each series) and the nondetection in the reproductive bees (lower graphs in each series). No viral proteins were detected in colony 3 at all (3D-images are exported from the Decyder 7.0 software, GE Healthcare).

GB19373, annotated as Dipeptidyl aminopeptidase III, is up-regulated in activated ovaries (ratio = -1.7 ; $p = 0.017$). This peptidase has been found to be both a cytosolic and membrane-bound hydrolase which targets, among others, proctolin at the Tyr-Leu bond.⁶⁰ Proctolin is a small neuropeptide, well-known in insects for its myotropic function⁶¹ and stimulatory effect on oviduct contraction.⁶² Although the peptide⁶³ and its receptor,⁶⁴ have not yet been demonstrated in *Apis mellifera*, it has been reported that queens start laying significantly more eggs and multiple eggs per cell upon proctolin injection.⁶⁵ However, the activity of Dipeptidyl aminopeptidase III might not be limited to the cleavage of proctolin alone. The presence of allatostatin^{66,67} and its receptor,⁶⁸ pheromone biosynthesis activating neuropeptides,⁶⁹ adipokinetic hormone (AKH)⁷⁰ and insulin-like peptides⁷¹ have been reported in insect ovaries. These peptides have honeybee orthologues and their sequences contain the Tyr-Leu, or the similar Tyr-Ile, cleavage-site; except for AKH. The presence of a protein convertase in activated ovaries might suggest that neuropeptides are regulators of ovary activation, as has been shown for allatostatin.^{72,73} Furthermore, allatostatin has been found to be up-regulated in sterile workers in a microarray comparing whole body RNA-profiles of sterile and reproductive workers.³⁸ We assume that the absence of a queen and brood are detected in the antennae and processed in the brain. This information is then transferred toward the ovaries through the hemolymph and might be mediated by (neuro)hormones, such as neuropeptides and steroid hormones.

Differential Viral Loads

Surprisingly, one of the spots with the highest up-regulation in rudimentary ovaries (ratio = 3.8 ; $p = 0.006$) is the polyprotein

of the Kakugo-virus (KV). Also, one of the two significantly differential spots in the brain experiment is the polyprotein of DWV (9-fold up-regulated in sterile workers, $p = 0.045$). Both KV and DWV are *picorna*-like viruses and KV is potentially linked to aggressive behavior in honeybees,^{74,75} whereas DWV is linked with wing deformations.⁷⁶ Due to the strong homology between those honeybee viruses, we are not able to discern whether the virus identified in our study is the Kakugo virus, Deformed wing virus or a third virus, the Varroa destructor virus. However, as more and more recombinants of those viruses are being identified (e.g., see refs 77–79), it becomes likely that they are variants of the same virus. Particularly interesting is that no viral proteins could be detected in the brains of both sterile and reproductive bees of the third colony (Figure 5).

Our proteomic data suggest that activated ovaries, including mature eggs, contain lower viral protein concentrations. DWV proteins are not even detected in brains of reproductive workers. However, it is highly intriguing that the findings of several proteomic experiments on several colonies and sample collections in different years are not validated at the RNA-level. Taken together, we found more viral proteins in the brain (9 replicates from 2 colonies), ovaries (4 replicates from 2 colonies) and the hemolymph (5 replicates from 2 colonies²⁵) of sterile workers. This finding is in agreement with a whole-body transcriptome analysis of 16 biological replicates, which reports that the GO term “viral assembly, maturation, egress and release” is significantly enriched in the sterile worker bees.³⁸

Additional evidence for the higher viral load in worker ovaries is the up-regulation of the aforementioned thioredoxin peroxidase 1 which is also linked to insect immune defense

against viruses.^{25,80} The fact that this potential immune system component is more abundant in the activated ovaries, might indicate that fertile workers are better able to control the RNA-virus, resulting in lower viral concentrations. The ovaries of infected queens, on the other hand, show signs of degeneration, although no decreased fertility was observed.⁸¹

From the qRT-PCR data, we can conclude that almost every bee is infected with DWV, and approximately equal percentages of reproductive and sterile bees show highly elevated virus titers. One could speculate that the viral protein detected in the proteomics experiments is due to a pooling effect in which only a small number of severely infected bees in a pool cause the differential viral load. This is, however, very unlikely based on the finding that highly infected bees include sterile as well as reproductive bees. Consequently, one would expect that the chance of sampling a severely infected bee in a pool is equal (50%) for both sterile and reproductive bees. For the brain experiment, this implies that the chance that our finding is caused by such a coincidence is less than 0.2% (0.5⁹) when we take only the two colonies (nine replicates) into account in which we detected the virus (Figure 5).

DWV and other highly similar viruses have already been reported in honeybee brains.^{82,83} It has been shown that infected bees have learning deficits in certain settings.⁸⁴ In another study comparing sterile and reproductive bees in a queenless colony, it has been suggested, based on transcriptional data, that sterile workers may start to forage earlier than reproductive bees.³⁸ One might wonder whether this early onset of foraging is induced by the virus. Theory predicts that worker bees will remove themselves from the colony, by performing riskier tasks, in order to protect the rest of the colony.^{85,86} It is tempting to speculate that the early foraging and learning deficits of the infected bees might increase the fitness of the virus itself. When an infected bee gets lost and manages to intrude into another colony, the virus may be transmitted to another colony. It is conceivable that the virus actively steers honeybee behavior, given that it has been found in the brain.⁸²

Several reasons can be put forward to explain the difference between the outcomes of our qRT-PCR and proteome experiments. First, our RNA and protein extracts, unfortunately, were not obtained from the same individuals, which could have delivered additional insight in this matter. The present qRT-PCR experiments have been carried out with bees obtained from another apiary in a different year and on worker bees which are the offspring of naturally mated queens, instead of singly mated (instrumentally inseminated) queens that were used in the proteomics experiment. However, the impact of those factors is likely minimal, as a similar approach has already proven to be successful.³⁸ Second, the qRT-PCR validation for the ovaries were performed on complete abdomens, instead of dissected ovaries. Third, the experimental colonies did not contain an unusual high number of parasitic mites or bees with deformed wings and no bees with deformed wings were included in the analysis. We are dealing with covert infections in both sterile and reproductive bees. Yet, it might be possible that the infections in reproductive bees are rather persistent, lacking viral replication, while in the sterile workers, the virus is actively replicating (for nomenclature see⁷⁶). The active replication of the virus requires that the RNA genome of DWV is accessible for translation and that the proteins have not (yet) formed a solid capsid protecting the viral genome. Continuing on this line of reasoning, it might be possible that

our lysis solution for proteomics is not able to separate the viral proteins from a solid virus particle and only solubilizes free viral proteins, whereas the phenol-based RNA extraction does successfully break down the viral capsids and releases the viral RNA. The virus particles are known to be resistant to numerous factors, as even isolated virus particles keep their virulence.⁸⁷ By consequence, via proteomics, we could actually be assessing viral replication while we determine complete viral loads using qRT-PCR. This is however, highly speculative and is definitely worth investigating in the future.

CONCLUSIONS

This work presents a 2DE spot map of both activated and inactivated honeybee worker ovaries. The data serve as a solid base for future research on reproductive regulation in social insects. The quantitative information provides insights in reproductive physiology, with homologies in vertebrates. Our experimental setup in a queenless colony provides a unique opportunity for research on the regulation of reproduction by comparing sterile and reproducing sisters of the same age, which is nearly impossible for most vertebrate model organisms. The observation of the DWV infection opens new avenues for the study of DWV reproduction and honeybee immunity.

ASSOCIATED CONTENT

Supporting Information

Supplemental tables and figure. This material is available free of charge via the Internet at <http://pubs.acs.org>.

AUTHOR INFORMATION

Corresponding Author

*Naamsestraat 59 B-3000, Leuven, Belgium, dries.cardoen@bio.kuleuven.be, Tel +32 16 32 39 44, fax +32 16 32 39 02.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

2-DE, 2-dimensional electrophoresis; 2D-DIGE, 2-dimensional differential gel electrophoresis; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-propane-sulfonate; DTT, dithiothreitol; DWV, deformed wing virus; FDR, false discovery rate; FE, fold enrichment; GO, gene ontology; Hsp, heat shock protein; KV, Kakugo virus; PMF, peptide mass fingerprinting; qRT-PCR, quantitative real-time polymerase chain reaction; RGPH, reproductive ground plan hypothesis; VP, Viral protein

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