Hemolectin expression reveals functional heterogeneity in honey bee (*Apis mellifera*) hemocytes

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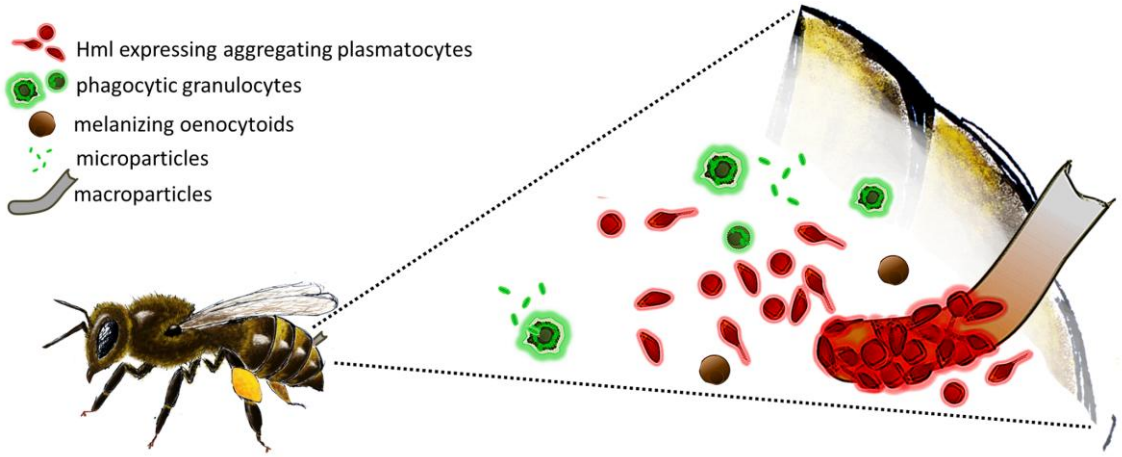
ABSTRACT

The identification of molecular markers considerably facilitated the classification and functional analysis of blood cell types. *Apis mellifera* hemocytes have been classified by morphological criteria and lectin binding properties; however, the use of molecular markers has been minimal. Here we describe a monoclonal antibody to a non-phagocytic subpopulation of *A. mellifera* hemocytes and to a constituent of the hemolymph clot. We demonstrate that the antibody identifies the *A. mellifera* hemolectin, a protein carrying human von Willebrand factor homology domains, characteristic of proteins involved in blood coagulation and platelet aggregation in mammals. Hemolectin expressing *A. mellifera* hemocytes contain the protein as cytoplasmic granules and contribute to the formation of a protein matrix, building up around foreign particles. Consequently, hemolectin as a marker molecule reveals a clear functional heterogeneity of hemocytes, allowing for the analytical separation of hemocyte classes, and could promote the molecular identification of hemocyte lineages in *A. mellifera.*



1. Introduction

Insects have inborn structures and mechanisms for defense against infection by parasites and microorganisms. The system is based on evolutionarily conserved signaling pathways and mechanisms, termed innate immunity (Hoffmann et al., 1999, Hultmark, 2003). Wounding, or pathogens entering the body upon septic injury, immediately trigger the activation of proteolytic cascades, leading to coagulation of hemolymph proteins, clot formation and melanization (Cerenius and Söderhäll, 2011, Theopold et al., 1998). The clot prevents the loss of body fluids and stops the spreading of microorganisms into the hemocoel by immobilizing the bacteria at the wound site (Bidla et al., 2005). Microbes and parasites entering the insect's body are recognized by blood cells termed hemocytes, the effector cells of cellular immunity, circulating in the hemolymph or residing as sessile cells in different tissues (Lanot et al., 2001, Lavine and Strand 2002, Márkus et al., 2009, Zettervall et al., 2004). Recognition of pathogens triggers hemocyte spreading (Gillespie et al., 1997, Williams et al., 2005) which promotes phagocytosis of microbes or encapsulation of larger foreign particles (Eleftherianos et al. 2009).

Insect hemocytes have been classified on the basis of their morphological, histochemical and functional characteristics (Gupta 1986, Jiravanichpaisal et al., 2006, Lavine and Strand 2002), as granular cells, plasmatocytes, spherule cells, oenocytoids and prohemocytes. The best studied organism is *Drosophila melanogaster* where three main classes of hemocytes have been described, plasmatocytes, crystal cells and lamellocytes. The plasmatocytes are the phagocytes, but besides engulfing microbes they produce antimicrobial peptides, extracellular matrix proteins and blood clotting components (Goto et al., 2001). In response to parasitic wasp infection special cell types are formed: the lamellocytes in *D. melanogaster* (Rizki and Rizki 1992), the nematocytes in *Zaprionus indianus* (Kacsoh et al., 2014) and the multinucleated giant hemocytes in spp. of the *ananassae* subgroup of Drosophilidae (Márkus et al., 2015). A minor population of the blood cells—called crystal cells in *Drosophila* (Meister, 2004), and named as oenocytoids in Lepidoptera (Lavine and Strand 2002)—secrete components of the phenoloxidase cascade and are involved in the melanization reaction. The classification of hemocytes initially relied on morphological criteria (Rizki, 1957). Later, the identification of different hemocyte classes was facilitated by the development of enhancer trap lines (Rodriguez et al., 1996), identification of immune system and response genes, novel mutations causing melanotic tumor formation in *D. melanogaster* (Braun et al., 1997), and by

the generation of monoclonal antibody libraries for the different hemocyte classes (Kurucz et al., 2003, Kurucz et al 2007a, Kurucz et al 2007b, Rus et al., 2006, Vilmos et al., 2004). These antibodies were also instrumental in the identification of molecules involved in regulating the cellular immune response.

Up to now, various methods have been employed in an attempt to identify the hemocyte subtypes of *Apis mellifera*. These included dye-staining methods and lectin binding assays to characterize honey bee hemocytes. Based on histochemical staining, size and morphological features they were classified into several subtypes, as phagocytic granular cells, plasmatocytes, oenocytoids, coagulocytes, binuclear cells, permeabilized cells and prohemocytes (de Graaf et al., 2002, El-Mohandes et al., 2010, Marringa et al., 2014, Negri et al., 2014, Schmid et al., 2008, Van Steenkiste 1988). Hemocytes were divided into subtypes by morphological features, ultrastructural characteristics and lectin binding properties as plasmatocytes—representing the major population (90%) of hemocytes in the circulation, subdivided to four subtypes P1-P4 on the basis of size—and a minor population of other cells, as prohemocytes, granular cells, oenocytoids and coagulocytes (de Graaf et al., 2002, Van Steenkiste 1988). Giemsa staining revealed five hemocyte types, as prohaemocytes, plasmatocytes, oenocytoids, granulocytes, coagulocytes and binucleated cells (El-Mohandes et al., 2010). The most abundant type (over 90%) was the plasmatoycte followed by granular cells and coagulocytes. Using the combination of flow cytometry and microscopy—hemocyte profiling—concluded that the *A. mellifera* hemolymph contains permeabilized cells, plasmatocytes and acellular objects that resemble microparticles (Marringa et al., 2014). Studies (Negri et al., 2014) using hemocytes of L5 stage *A. mellifera* larvae and newly emerged adult workers in *in vitro* experiments identified two cell types in the larva, and four cell types in the adult hemocytes by their attachment and spreading on solid substrates, agglomeration and phagocytosis. These data all represent of high relevance with respect to the homeostasis of the organism and cellular immunity to parasites and microbes, however, so far the use of molecular markers, including cell-type specific antibodies, for definition of honey bee hemocyte subsets has been minimal.

To address the issue of molecular and functional heterogeneity in honey bee hemocytes we took a combined immunological and functional approach in the hope that development of antibodies with a high specificity for functionally different hemocyte subsets will put cell typing on firm ground (de Graaf et al., 2002, Negri et al., 2015). Using the hybridoma

technology in combination with functional assays we aimed to resolve functional heterogeneity of hemocytes on the basis of the expression of a hemocyte specific antigen. We show that the use of a monoclonal antibody combined with functional assays is an effective way to identify different hemocyte subsets in the honey bee.

2. Materials and Methods

2.1 Laboratory animals, collection of hemocytes and hemolymph

Studies did not involve endangered or protected species. *Apis mellifera* worker adults and worker larvae were collected from colonies in an apiary in the Szeged-region (Hungary). None of the colonies or the experimental individuals showed symptoms of disease. Fourth-fifth stage worker larvae (L4-L5) were washed off from the brood with *Drosophila* Ringer's solution (130 mM NaCl, 5.0 mM KCl, 1.0 mM $CaCl_2$) and kept between 32-35°C for up to two hours until hemocyte sampling. The larvae and CO_2-anesthetized young adults were perfused with 100 µl of Schneider's insect medium supplemented with 2 mM L-glutamine (Sigma-Aldrich), 5% fetal bovine serum (FBS; GIBCO) and 0.01% 1-phenyl-2-thiourea (PTU) (Sigma-Aldrich,) and the hemolymph was collected in 1.5 ml Eppendorf centrifuge tubes on ice. The hemocytes were counted in a hemocytometer.

2.2 Production and screening of monoclonal antibodies

Monoclonal antibodies (mAb) (Köhler and Milstein, 1975, Köhler and Milstein, 1976) were raised against *A. mellifera* hemocytes as described previously for *D. melanogaster* (Kurucz et al., 2007b) and *D. ananassae* (Márkus et al., 2015). Briefly, BALB/C mice were immunized three times with 10^6 hemocytes in 1 ml *Drosophila* Ringer's solution, with three week intervals. Three days after the final boost spleen cells were fused with SP2/0 myeloma cells by using polyethylene glycol (PEG 1540, Sigma-Aldrich) and the fusion product was distributed in 96 well flat bottomed plates (Costar). Hybridoma culture supernatants were screened by immunohistochemistry on glass-adhered acetone fixed larval and adult hemocytes. The selected hybridomas were subcloned by limiting dilution. For the present study the 4E1, an IgG1κ antibody was used. The isotype of the antibody was determined by IsoStrip Mouse Monoclonal Antibody Isotyping Kit (Roche Diagnostics).

2.3 Immunohistochemistry (IH) and indirect immunofluorescence (IIF)

All procedures were carried out at room temperature. Hemocytes in Schneider's medium (60 µl) supplemented with 5% FBS and PTU were left to adhere on 12-spot microscope slides (Hendley-Essex: SM-011) for 60 min then fixed with acetone for 6 min, air dried, rehydrated and blocked with PBS (1.78 mM Na_2HPO_4, 5.1 mM Na_2HPO_4, 140 mM NaCl) containing 0,1% BSA (Roche) (PBS-BSA) for 20 min. Samples were incubated with the hybridoma culture supernatant for 1 h, washed three times for 5 min each, with PBS. For immunohistochemistry the samples were incubated with biotinylated goat anti-mouse antibody (1.46 mg/ml diluted 1:500 in PBS-BSA, DAKO) for 45 min, washed three times with PBS. Then the samples were incubated with Streptavidin HRPO (0.85 mg/ml diluted 1:300 in PBS-BSA, DAKO) and washed again three times with PBS. The samples were washed with sodium-acetate solution, (0.2 M, pH 4.6) three times, 3 min each and the reaction was visualized by 0.05% 3-amino-9-ethylcarbazole (AEC, Sigma-Aldrich). For visualization of the nuclei DAPI (Sigma 1:400) was used. For indirect fluorescence, as secondary antibodies, Alexa Fluor 568 or Alexa Flour 488 conjugated anti-mouse IgG (Invitrogen, 1:1000) were applied in PBS-BSA for 45 min, containing DAPI. The slides were washed three times with PBS and mounted in Fluoromount G (Southern Biotech). Live hemocytes were reacted with 4E1 antibody and Alexa Flour 568 conjugated anti-mouse IgG (Invitrogen, 1:1000) on ice. Larval cuticle and midgut were dissected in Schneider's medium on ice, fixed with acetone and reacted with the antibodies as described for hemocytes. Samples were examined with an epifluorescence microscope (Zeiss Axioskop 2 MOT) or an Olympus FV1000 confocal LSM microscope.

2.4 Phagocytosis assay

The animals were injected with 50 µl fluorescein isothiocyanate conjugated Gram negative *Escherichia coli* (SzMC 0582) (Szeged Microbial Collection, University of Szeged, Hungary) (FITC-*E. coli*) *Enterobacter cloacae* (SzMC 21890) (Szeged Microbial Collection, University of Szeged, Hungary), Gram positive *Melissococcus pluton* (National Food Chain Safety Office, Hungary) and *Staphylococcus aureus* (SzMC 0579) (Szeged Microbial Collection, University of Szeged, Hungary) bacteria as described in Kurucz et al., 2007a. The hemolymph was collected 45 min after the injection in Schneider's medium supplemented with 5% FBS and PTU. The hemocytes were adhered on spots of 12-spot microscope slides, and indirect immunofluorescence analysis was carried out using Alexa Fluor 568 conjugated anti-mouse IgG (Invitrogen, 1:1000) as a secondary antibody, as described previously.

2.5 Clot formation assay

The "hanging drop" method was used as described by Bidla et al., 2005. The bees were bled on a microscopic slide which was then turned upside down and incubated in a humid chamber. After 20 min the slide was reverted to normal position. Another slide was slowly lowered to the hemolymph drop avoiding complete contact and the clot was caught. The samples were fixed with acetone for 6 min for IH and IIF.

2.6 Encapsulation assay

To study the encapsulating activity of hemocytes (Andrade et al., 2010, Brandt et al., 2016, Cox-Foster and Stehr, 1994, Renault et al., 2002) a 0.08 mm nylon fishing line was cut into 5 mm pieces, sterilized with 75% alcohol and threaded through between the 3rd and the 4th abdominal segments of CO_2-anesthetized, immobilized adult workers. After 3 hours the plastic fibers were removed by dissecting the abdomen and placed on a microscopic slide, fixed with 2% paraformaldehyde for 15 min and visualized by IIF.

2.7 Immunoprecipitation

Ten milliliters of hybridoma supernatant was added to 50 µl of slurry Protein G Sepharose beads and rotated at room temperature for 1 h. The Protein G Sepharose-antibody beads were washed with borate buffered saline (0.2 M boric acid/NaOH pH 9.0) three times and rotated with 5.2 mg/ml dimethyl-pimelimidate-dihydrochloride (DMP, Sigma-Aldrich) in Borate buffered saline for 30 min. The beads were then washed two times with 0.2 M ethanolamine, rotated for 2 h with 0.2 M ethanolamine (pH 8) and washed three times with PBS. Hemocytes were isolated from the hemolymph, by centrifugation (4°C, 1800 rpm, 8 min). The cells were extracted in lysis buffer containing 50 mM Tris/HCl (Reanal) pH 8.0, 150 mM $MgCl_2$ (Reanal), 1% NP40 (Fluka), 5 mM EDTA (Sigma), 0.1% SDS (Sigma), 10 mM PMSF (Sigma) and Complete Protease Inhibitor Cocktail (Roche), according to the manufacturer's instructions, for one hour. Fifty µl (20% Protein G Sepharose) beads were mixed with 300µl hemocyte lysate and incubated overnight at 4°C. Then the beads were washed three times with lysis buffer and incubated in SDS-PAGE sample buffer (15.6 mM Tris/HCL pH 6.8, 6.25% glycerol, 0.5% SDS, 0.003% bromophenol blue) for one hour at 56°C and subsequently boiled for 5 min. The resulting samples were analyzed with SDS-PAGE/Western blot.

2.8 Western blot analysis

The immunoprecipitated proteins were separated by 5-7.5% SDS-PAGE under non-reducing conditions. The proteins were transferred from the gels onto a polyvinylidene fluoride (PVDF) membrane (Millipore) in transfer buffer (25 mM Tris, 90 mM glycine, 20% methanol). Transfer was performed at a constant voltage of 30 V, overnight at 4°C. Non-specific binding sites on the membrane were blocked for 1 h at room temperature with TBS (10 mM Tris/HCL pH 7.5, 150 mM NaCl) containing 5% fat-free milk powder. The blot was incubated with the hybridoma supernatant for 1.5 h with agitation at room temperature and washed with TBS three times for 10 min each and then incubated with the secondary HRPO-conjugated anti-mouse IgG (DAKO, 1:5000 in TBS-1% BSA). After three washes in TBS, 10 min each, the reaction was visualized by the ECL-Plus system (GE Healthcare).

2.9 Silver staining and LC-MS/MS analysis

The immunoprecipitated proteins were separated by 5-7.5% SDS-PAGE under non-reducing conditions. The gel was incubated in fixing solution I. (50% methanol, 12% acetic acid, 0.019% formaldehyde) for 1 h and washed three times, 10 min each, with 50% ethanol. The gel was sensitized with 0.02% sodium thiosulfate (0.02 g $Na_2S_2O_3$, 100 ml H_2O) for 2 min, washed three times with distilled water, 20 sec each, and incubated with 0.2% silver nitrate solution (0.2 g $AgNO_3$, 100 ml H_2O) for 20 min in the dark. After washing the gel with distilled water three times, 20 sec each, the reaction was developed with a sodium carbonate solution (6 g Na_2CO_3, 40 µl of 1% $Na_2S_2O_3$ and 53 µl of 35% formaldehyde in 100 ml distilled water). The reaction was terminated with two washes with distilled water, 20 sec each, and the gel was incubated in fixing solution II. (50% methanol, 12% acetic acid in distilled water) for 10 min. The high molecular weight band corresponding to the Western blot signal was excised from the gel, digested with trypsin and analyzed by mass spectrometry with online LC-MS/MS as described in the Supplementary material section. As a control, another aliquot of the hemocyte extract was incubated with irrelevant, isotype matched antibody coated Protein G Sepharose beads, and the immunoprecipitated material analyzed in parallel.

2.10 Synthesis of double-stranded RNA (dsRNA) and RNA interference

RNA was isolated from *A. mellifera* adult workers to generate cDNA, which was used as template for the amplification of a 559 bp *A. mellifera hemolectin (AmHml)* specific fragment. In the reaction the 5'-

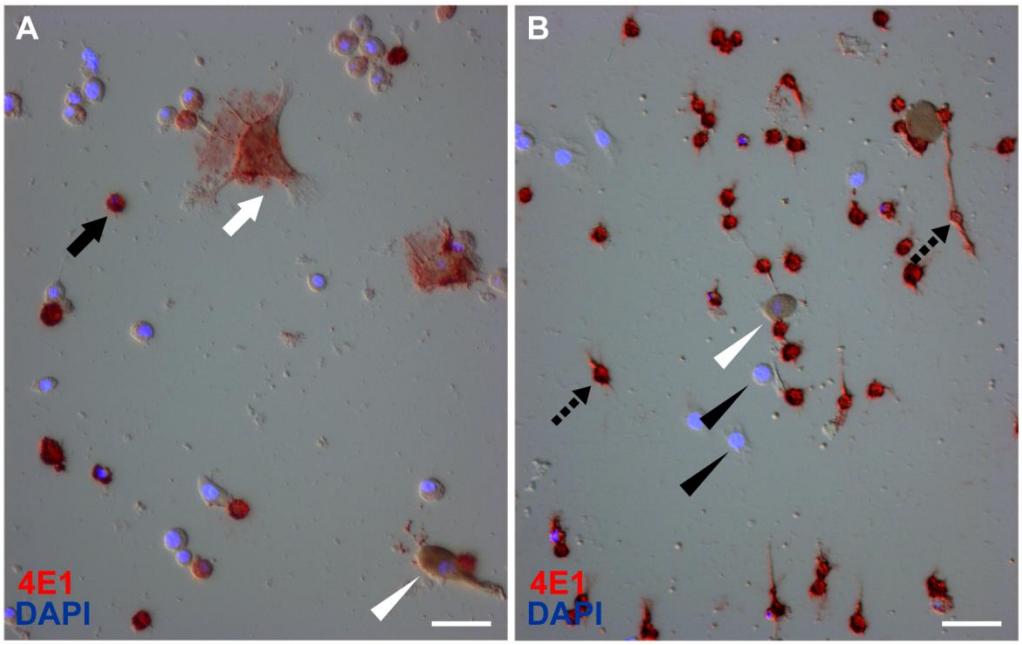
AGTTAATACGACTCACTATAGGAGTAACCATCAAGAAATAAC-3' and 5'-AGTTAATACGACTCACTATAGGGTCTTTCCTCTGGTTAAAAC-3' primer pair was used (T7 adapter sites are underlined). A GFP carrying pBluescript vector was used and the 5'-ATTTAATACGACTCACTATAGGTGCTTTTCAAGATACCCAGATC-3' and 5'-ATTTAATACGACTCACTATAGGTTCATCCATGCCATGTGTAATC-3'primer pair to amplify a 542 bp control fragment. Fragments were purified with the EZ-10 Spin Column PCR Products Purification Kit (Bio Basic), and DNA sequence analysis was done using a BigDye Terminator v3.1 Cycle Sequencing Kit (Invitrogen) and a 3500-Genetic Analyzer (Applied Biosystems). 1000 ng of the obtained *hml* and GFP specific fragments were used as templates in dsRNA synthesis, which was done using T7 RiboMax Express RNAi System (Promega) kit. The obtained dsRNA was used for gene silencing as described by Nunes and Simões (2009). Briefly, second instar larvae were fed with 1 µl of sucrose solution containing 1.5 µg of AmHml-dsRNA inside the worker brood cells. One control group was fed with 1 µl sugar solution containing 1.5 µg dsGFP-RNA, another control group was left without treatment. Two hours after dsRNA treatment all combs were returned to the original colony to allow the development of the larvae under natural conditions until capping. Reaching the last larval stage (L5) the larvae were collected, the hemocytes were harvested, indirect immuofluorescence staining was performed using the 4E1 antibody and the proportion of the fluorescent cells of individual larvae was determined. Approximately 1000 cells were counted in each sample. The results of four independent experiments were summarized. Data were analyzed using one-way ANOVA statistics to verify differences between groups and the level of significance was determined with Tukey HSD (significance at $p \leq 0.05$) in SPSS Statistics 17.0.

3. Results

3.1 The 4E1 antibody reacts with a subpopulation of *A. mellifera* hemocytes

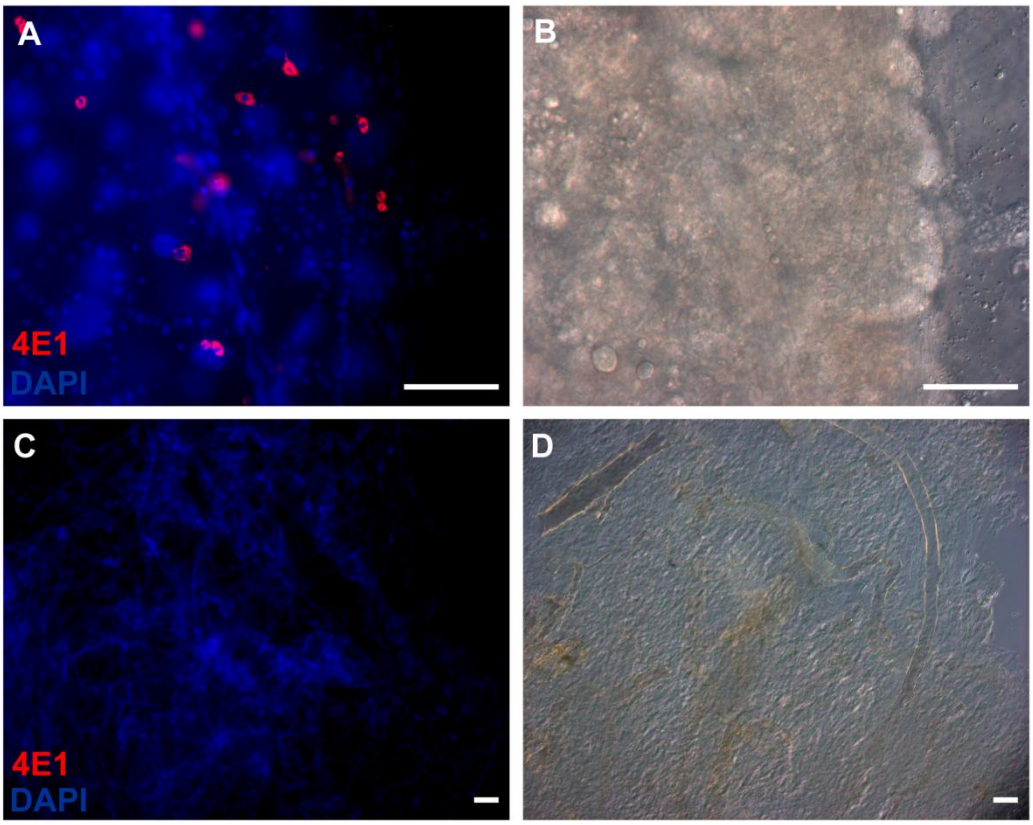
We developed monoclonal antibodies (mAb) by immunization of mice with hemocytes of adult workers. We observed that one of them, the 4E1 mAb, reacted with a subpopulation of larval and adult hemocytes. In fifth stage (L5) larvae it reacted with about 30% of circulating hemocytes: a fraction of spherical cells (Fig. 1A black arrow) and with large cells having polygonal morphology (Fig. 1A white arrow). In the young adult it reacted with 80% of the circulating hemocytes, the cells were spherical and had fusiform extensions (Fig. 1B dashed

arrows), but was unreactive with big spherical, small polygonal cells (Fig. 1B black triangles). It was also unreactive with melanizing cells (Fig. 1A, 1B white triangles).



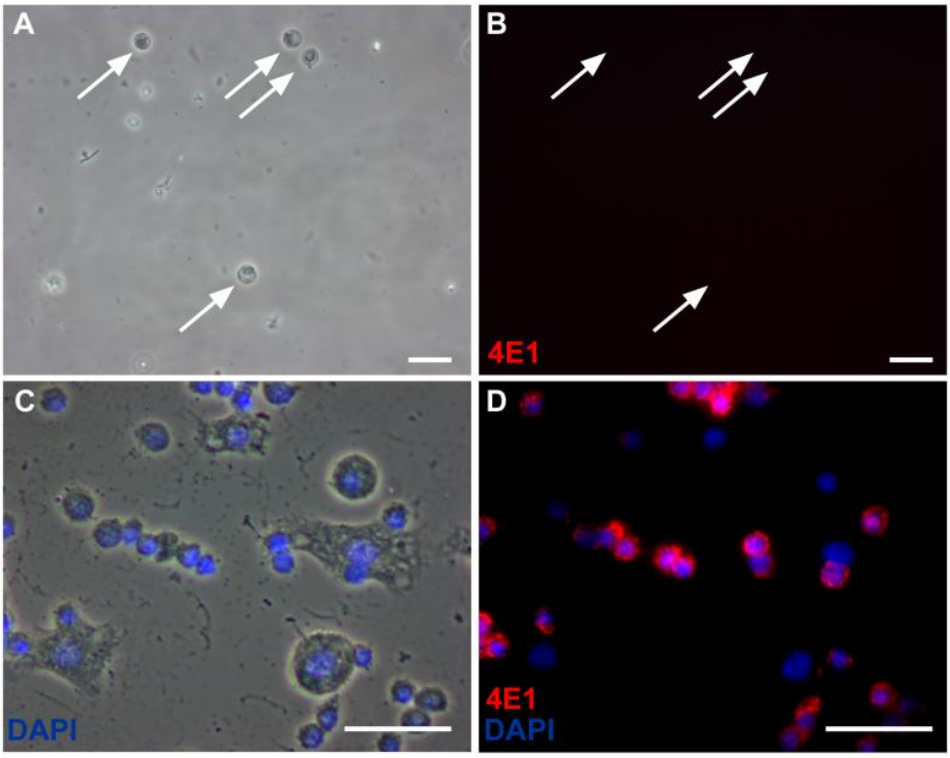
1. Figure The reaction of the 4E1 antibody with circulating larval (A) and adult (B) hemocytes. Nomarski image of the immunohistochemical reaction (red) of acetone fixed hemocytes after 4E1 mAb/anti-mouse IgG-HRPO/AEC staining. In the fifth stage (L5) larvae (A) the 4E1 antibody reacts with a fraction of spherical cells (black arrow) and with large cells with polygonal morphology (white arrow). In the adult (B) it reacts with spherical cells with fusiform extensions (dashed arrows), but unreactive with large spherical and polygonal cells (black triangles). It is also unreactive with the melanizing cells (white triangles). Nuclei were visualized with DAPI staining (blue). The scale bars represent 20 μm.

Immunostaining of cuticle (Fig. 2A, 2B) and midgut (Fig. 2C, 2D) preparations revealed a reaction with small cells scattered on the inner surface of the cuticle (Fig. 2A). These hemocytes were apparently attached to the wall of the haemocoel. Other structures in the cuticle or in the midgut (Fig. 2C) did not stain, confirming that the expression of the antigen recognized by the 4E1 antibody is restricted to hemocytes.



2. Figure The 4E1 antibody reaction is restricted to the hemocytes. Reaction of the 4E1 antibody with larval cuticle (A) and with adult midgut (C) visualized by indirect immunofluorescence. Nomarski images of the cuticle (B) and the midgut (D) preparations. The 4E1 mAb/anti-mouse IgG-Alexa Fluor 568 staining is showing up as red on the hemocytes attached to the epithelial layer of the haemocoel (A). Other structures in the cuticle or in the midgut did not stain. The nuclei are stained with DAPI (blue). The scale bars represent 40 μm.

In order to determine the cellular localization of the immunoreaction we performed indirect immunofluorescence staining of native and acetone-fixed permeabilized adult hemocytes. Live blood cells (Fig. 3A arrows) did not show cell membrane staining (Fig. 3B arrows), but in a subpopulation of fixed permeabilized hemocytes granular cytoplasmic staining (Fig. 3D) was detected.

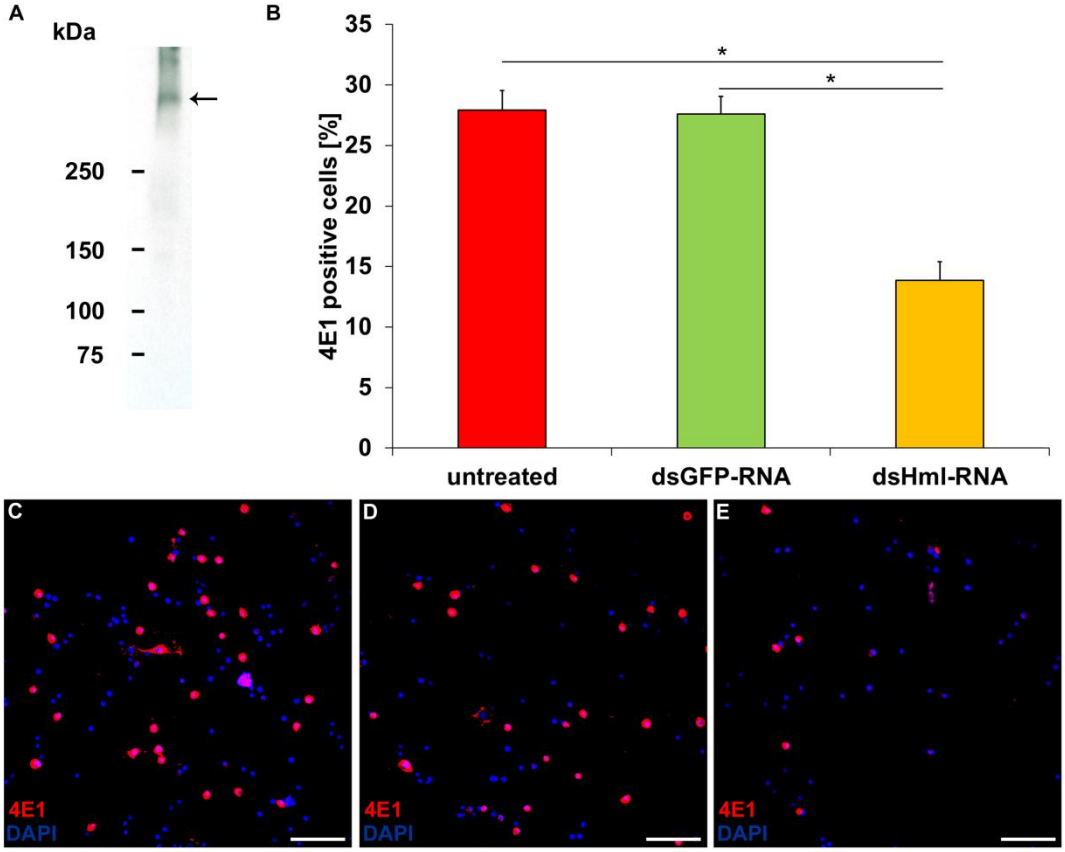


3. Figure The expression of the 4E1 marker in circulating adult hemocytes. Live cells in suspension (A, B arrows) and adhered-fixed-permeabilized hemocytes(C, .D) were reacted with the 4E1 mAb and anti-mouse IgG-Alexa Fluor 568 (red). The nuclei were stained with DAPI (blue), the scale bars represent 20 μm.

3.2 The 4E1antigen is encoded by the *A. mellifera hemolectin (AmHml)* gene

To determine the molecular mass of the antigen recognized by the 4E1 antibody, we performed Western blot analysis of hemocyte extract by SDS-PAGE using a non-reducing gel system. We detected a protein band with an approximate molecular mass of 400 kDa (Fig. 4A). The antigen was immunoprecipitated as described above and a slot corresponding to the Western blot signal was isolated. The LC-MS/MS (Supplementary data) analysis identified a 420 kDa *A. mellifera* protein (Uniprot UD: A0A087ZSR0) with high confidence (with 14 unique peptides providing 5% sequence coverage), which showed high homology to hemocytin/hemolectin of other insects, including the *Melipona quadrifasciata* stingless bee (Uniprot ID: A0A0N0BH61), the *Habropoda laboriosa* bumble bee (Uniprot ID: A0A0L7QSW0) and the *Camponotus floridanus* ant (Uniprot ID: E2AVY8). To verify that the 4E1 molecule was encoded by the *A. mellifera hemolectin* gene (*AmHml*) (Lesch, 2007, Wallberg et al., 2014) we silenced the gene transcript in larvae with a non-invasive method (Nunes and Simões 2009). In short, for RNA interference we synthetized *AmHml* specific double-stranded RNA (AmHml-dsRNA) and fed L2 larvae with a sucrose solution containing AmHml-dsRNA inside the worker brood cells. Larvae exposed to GFP-dsRNA and untreated larvae were used as controls. At the L5 larval stage the larvae were bled and hemocytes were

stained by indirect immunofluorescence using the 4E1 antibody. The proportion of the 4E1 expressing hemocytes in the AmHml-dsRNA treated larvae was lower (14%) (Fig. 4B, 4E) than in the GFP-dsRNA treated (28%) (Fig. 4B, 4D) or in the untreated (28%) controls (Fig. 4B, 4C) ($p<0.05$). However, 4E1 molecule was still present in a minor population of hemocytes in the AmHml-dsRNA treated animals (Fig. 4B, 4E). Possibly, these cells already express 4E1 molecule at the larval L2 stage, prior to AmHml-dsRNA treatment. On the other hand, dsRNA expression often leads to incomplete suppression of gene transcripts.

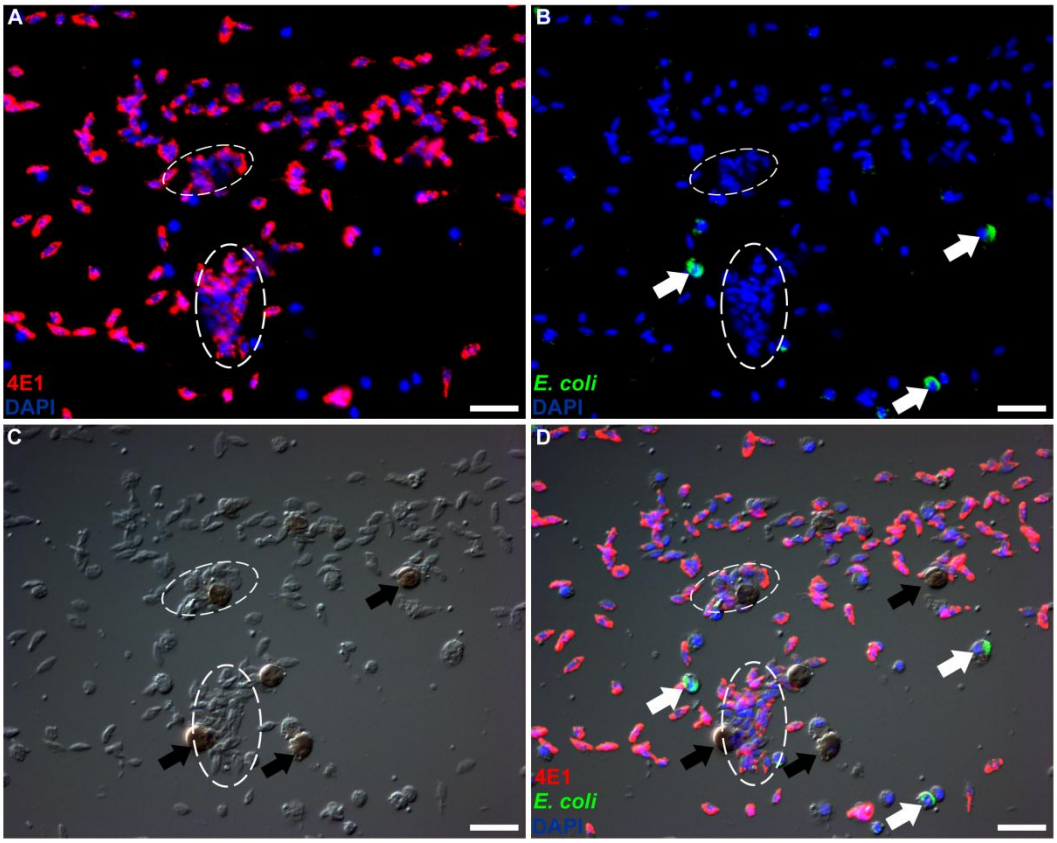


4. Figure Western blot analysis of the 4E1 marker and RNA interference. A: Western blot analysis of the hemocyte lysate using the 4E1 antibody. Arrow points to the position of the Hml band. B-E: RNAi analysis of the *AmHml* gene. The percentage of the 4E1 marker positive cells was determined in the AmHml-dsRNA (B yellow bar, E red staining) the GFP-dsRNA (B green bar and D red staining) and the untreated (B red bar, C red staining) groups. Data were analyzed using one-way ANOVA statistics followed by Tukey's post hoc test ($p < 0.05$). The error bars represent the standard error of the mean. The nuclei were stained with DAPI (blue). The scale bars shows 50 μm.

3.3 Hemolectin expression resolves functional heterogeneity of hemocytes

As AmHml is expressed in a subset of hemocytes we carried out combined functional and serological analysis by correlating the AmHml expression with phagocytic capacity of the cells. We injected fluorescein isothiocyanate conjugated *Escherichia coli* (FITC-*E. coli*) into

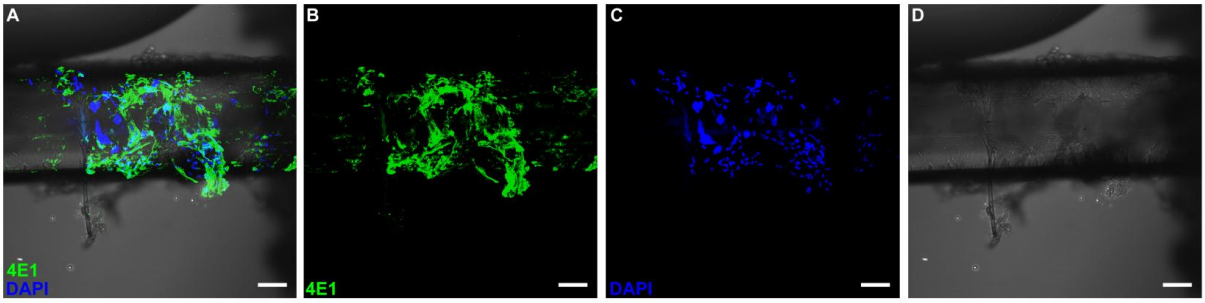
young adults, bled the animals on slides after 45 min, let the cells adhere and stained them with 4E1 antibody. As shown in Fig. 5 the majority of circulating hemocytes expressed AmHml. The AmHml positive cells formed aggregates (Fig. 5) and did not engulf FITC-*E.coli* bacteria (Fig. 5B, 5D). Phagocytosis assay was carried out with FITC conjugated *Enterobacter cloacae Melissococcus pluton* and *Staphylococcus aureus* bacteria too, with the same result (not shown). Analysis of the correlation between melanization (Fig. 5C) and AmHml expression (Fig. 5A) revealed that AmHml expressing cells did not melanize and melanizing oenocytoids did not express AmHml (Fig. 5D). The AmHml expressing cells were also not involved in the clearance of bacteria (Fig. 5B, 5D).



5. Figure Fluorescent and Nomarski pictures of phagocytosis, melanization and aggregation of adult hemocytes. The hemocytes were isolated from FITC-E.coli (green) injected adults, adhered to microscopic slide, fixed and reacted with 4E1 and Alexa Fluor 568 conjugated anti-mouse IgG antibody (red). The nuclei are visualized with DAPI (blue). The 4E1 reactive aggregates are encircled with dashed ovals (A-D), phagocytic cells are indicated by white arrows (B, D) and black arrows point to melanizing cells on Nomarski image (C, D). "D" is the merged image. The scale bars represent 20 μm.

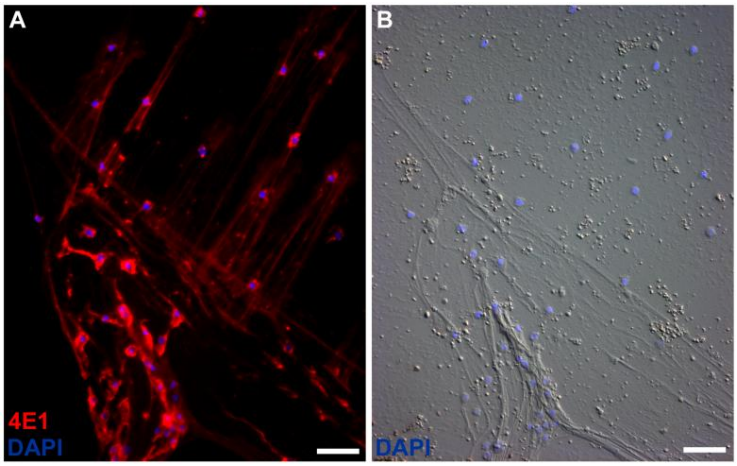
The adhesion to foreign particles, and the encapsulation reaction (Andrade et al., 2010, Brandt et al., 2016, Cox-Foster et al., 1994, Renault et al., 2002) was modeled by inserting a 0.08 mm diameter nylon fiber between the third and the fourth abdominal segments (Fig. 6). The fiber was removed after 3 hours and stained for the presence of AmHml. We found that AmHml

positive cells were embedded into an AmHml positive matrix as a clot formed around the plastic fiber (Fig. 6A).



6. Figure Encapsulation reaction. Plastic fiber was threaded through between the 3rd and the 4th abdominal segments of the adult. After three hours incubation the plastic fiber was carefully removed, fixed and stained with the 4E1 mAb and anti-mouse IgG-Alexa Fluor 488 (green) (A, B). The nuclei were stained with DAPI (blue) (A, C). Nomarski image (D). Merged picture (A).The scale bars represent 20 µm.

As hemocyte aggregates and clots are frequently formed spontaneously on the glass microscope slides after bleeding we checked for the presence of AmHml in the aggregates. Immunostaining of hemocyte smears revealed that AmHml is a component of the clot in which AmHml positive hemocytes were embedded. We also induced clot formation by the "hanging drop" method (Bidla et al., 2005), isolated the clot and stained it for the presence of AmHml. We detected AmHml in the isolated clot fibers and also revealed the presence of entrapped AmHml positive hemocytes in the aggregates (Fig. 7).



7. Figure Indirect immunofluorescence staining of the hemolymph clot (Bidla *et al*., 2005). The clot was stained with indirect immunofluorescence by using the 4E1 mAb/ anti-mouse IgG-Alexa Fluor 568 (red) (A), Nomarski image (B). The cell nuclei were stained with DAPI (blue), the scale bars represent 20 µm.

4. Discussion

The aim of our studies was to uncover molecular and functional heterogeneity of honey bee hemocytes. We developed a mAb and showed that it reacts with an intracellular molecule in a subpopulation of blood cells. By Western blot analysis we detected a protein with a molecular mass corresponding to the *A. mellifera* hemolectin (AmHml) and mass spectrometry analysis confirmed that the protein is the AmHml. To verify that the mAb recognizes AmHml we performed a non-invasive RNA interference (Nunes and Simões 2009) with *AmHml* specific double-stranded RNA and observed substantial reduction in the number of hemocytes expressing the molecule reacting with 4E1 antibody.

Hemolectin is the major hemocyte derived component in the hemolymph soft clot, which possess von Willebrand factor homology domains, characteristic for proteins involved in blood coagulation and platelet aggregation in mammals (Goto et al., 2003, Lesch et al., 2007). The *hemolectin (Hml)* homolog *hemocytin* (*Hmc*) gene has been described from *Bombyx mori* (Kotani et al., 1995) and a *Hml* related gene was identified in the genome of *A. mellifera* (Consortium, 2006, Lesch et al., 2007). The honey bee hemolectin could be an important factor in defense against *Varroa* infections, where coagulation and clot formation is crucial to prevent blood loss and the entry of other pathogens. African and European bees have differences in the survival of *Varroa* infections (Martin and Medina, 2004) and a further study showed that along with other immune genes *AmHml* differs between these groups (Wallberg et al., 2014).

In contrast to *Drosophila*, where a subset of hemocytes express the Hml molecule and the majority of both larval and adult hemocytes called plasmatocytes are phagocytic cells (Williams 2007), in honey bee both in the larva and in the adult we observed a clear heterogeneity and complementarity with respect to phagocytosis and AmHml expression. In the larva the majority of the circulating hemocytes exhibit phagocytic function, while AmHml is expressed in about 30% of the hemocytes. In the young adult 80% of circulating hemocytes express AmHml and a non-overlapping 15-20% of hemocytes are phagocytic. As there is no overlap between phagocytosis and AmHml expression it is conceivable that there is a functional heterogeneity within circulating hemocytes (e.g. a minor population exhibits phagocytic function while the majority is involved in the production of a coagulation factor) revealed by antibody staining. This dichotomy of cell populations is supported by our finding

that the majority of hemocytes in the hemolymph coagulum and the cells involved in the demarcation and encapsulation of the foreign particles are the AmHml expressing hemocytes. In circulation we also distinguished a third, minor hemocyte population. This minor population consists of large spherical cells, lacking AmHml expression and phagocytic capacity. With light microscopy these cells generally show brownish color as an indication of melanization reaction. These cells are apparently those defined as the oenocytoids, involved in the melanization of decaying tissues and in blood clotting (Cousin et al., 2013).

On the basis of these studies we anticipate that antibodies with high specificity to functionally different cell types can be developed and used for the identification and characterization of honey bee hemocyte subsets and lineages. This method has proven to be invaluable for understanding the function of hemocyte lineages in other insects (Arai et al., 2013, Kurucz et al., 2007b). Studies of the mechanisms of cell mediated immunity may contribute to the development of strategies to reduce or prevent the harm caused by the recent spreading of infectious diseases, pests and the damage caused by multiple factors associated with the consequences of "factory farming", where thousands of families are kept in one location (Bull et al., 2012, Chan et al., 2009, Evans and Schwarz, 2011, Farooqui, 2013, Simon-Delso et al., 2014, vanEngelsdorp et al., 2009).

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References

Andrade, G.S., Serrão, J.E., Zanuncio, J.C., Zanuncio, T.V., Leite, G.L.D., Polanczyk, R.A., 2010. Immunity of an Alternative Host Can Be Overcome by Higher Densities of Its Parasitoids *Palmistichus elaeisi*s and *Trichospilus diatraeae*. PLoS One 5. doi:10.1371/journal.pone.0013231

Arai, I., Ohta, M., Suzuki, A., Tanaka, S., Yoshizawa, Y., Sato, R., 2013. Immunohistochemical analysis of the role of hemocytin in nodule formation in the larvae of the silkworm, *Bombyx mori*. J. Insect Sci. 13, 125. doi:10.1673/031.013.12501

Bidla, G., Lindgren, M., Theopold, U., Dushay, M.S., 2005. Hemolymph coagulation and phenoloxidase in *Drosophila* larvae. Developmental & Comparative Immunology 29, 669–679. doi:10.1016/j.dci.2004.11.007

Brandt, A., Gorenflo, A., Siede, R., Meixner, M., Büchler, R., 2016. The neonicotinoids thiacloprid, imidacloprid, and clothianidin affect the immunocompetence of honey bees (*Apis mellifera L.*). J. Insect Physiol. 86, 40–47. doi:10.1016/j.jinsphys.2016.01.001

Braun, A., Lemaitre, B., Lanot, R., Zachary, D., Meister, M., 1997. *Drosophila* immunity: analysis of larval hemocytes by P-element-mediated enhancer trap. Genetics 147, 623–634.

Bull, J.C., Ryabov, E.V., Prince, G., Mead, A., Zhang, C., Baxter, L.A., Pell, J.K., Osborne, J.L., Chandler, D., 2012. A Strong Immune Response in Young Adult Honeybees Masks Their Increased Susceptibility to Infection Compared to Older Bees. PLoS Pathog 8, e1003083. doi:10.1371/journal.ppat.1003083

Cerenius, L., Söderhäll, K., 2011. Coagulation in invertebrates. J Innate Immun 3, 3–8. doi:10.1159/000322066

Chan, Q.W.T., Melathopoulos, A.P., Pernal, S.F., Foster, L.J., 2009. The innate immune and systemic response in honey bees to a bacterial pathogen, *Paenibacillus larva*e. BMC Genomics 10, 387. doi:10.1186/1471-2164-10-387

Cousin, M., Silva-Zacarin, E., Kretzschmar, A., El Maataoui, M., Brunet, J.-L., Belzunces, L.P., 2013. Size changes in honey bee larvae oenocytes induced by exposure to Paraquat at very low concentrations. PLoS ONE 8, e65693. doi:10.1371/journal.pone.0065693

Cox-Foster, D.L., Stehr, J.E., 1994. Induction and localization of FAD-glucose dehydrogenase (GLD) during encapsulation of abiotic implants in *Manduca sexta* larvae. Journal of Insect Physiology 40, 235–249. doi:10.1016/0022-1910(94)90047-7

de Graaf, D.C. de, Dauwe, R., Walravens, K., Jacobs, F.J., 2002. Flow cytometric analysis of lectin-stained haemocytes of the honeybee (*Apis mellifera*). Apidologie 33, 571–579. doi:10.1051/apido:2002041

Eleftherianos, I., Xu, M., Yadi, H., Ffrench-Constant, R.H., Reynolds, S.E., 2009. Plasmatocyte-spreading peptide (PSP) plays a central role in insect cellular immune defenses against bacterial infection. J. Exp. Biol. 212, 1840–1848. doi:10.1242/jeb.026278

El-Mohandes S.S., Nafea, E.A., Fawzi, A.M., 2010. Effect of different feeding diets on the haemolymph of the newly emerged honeybee workers *Apis mellifera L*. Egypt. Acad. J. Biolog. Sci., 3 (1), 213 - 220

Evans, J.D., Schwarz, R.S., 2011. Bees brought to their knees: microbes affecting honey bee health. Trends Microbiol. 19, 614–620. doi:10.1016/j.tim.2011.09.003

Farooqui, T., 2013. A potential link among biogenic amines-based pesticides, learning and memory, and colony collapse disorder: a unique hypothesis. Neurochem. Int. 62, 122–136. doi:10.1016/j.neuint.2012.09.020

Gillespie JP, Kanost MR, Trenczek T.1997. Biological mediators of insect immunity. Annu Rev Entomol. 42:611-43.

Goto, A., Kadowaki, T., Kitagawa, Y., 2003. *Drosophila* hemolectin gene is expressed in embryonic and larval hemocytes and its knock down causes bleeding defects. Dev. Biol. 264, 582–591.

Goto, A., Kumagai, T., Kumagai, C., Hirose, J., Narita, H., Mori, H., Kadowaki, T., Beck, K., Kitagawa, Y., 2001. A *Drosophila* haemocyte-specific protein, hemolectin, similar to human von Willebrand factor. Biochem. J. 359, 99–108.

Gupta, A.P. 1986. Arthropod immunocytes. In: Hemocytic and Humoral Immunity in *Arthropods,* pp. 13-59 (A.P. Gupta, ed.). John Wiley and Sons, New York 3-59.

Hoffmann, J.A., Kafatos, F.C., Janeway, C.A., Ezekowitz, R.A., 1999. Phylogenetic perspectives in innate immunity. Science 284, 1313–1318.

Honeybee Genome Sequencing Consortium, 2006. Insights into social insects from the genome of the honeybee *Apis mellifera*. Nature *443*, 931–949.

Hultmark, D., 2003. *Drosophila* immunity: paths and patterns. Curr. Opin. Immunol. 15, 12–19.

Jiravanichpaisal, P., Lee, B.L., Söderhäll, K., 2006. Cell-mediated immunity in arthropods: hematopoiesis, coagulation, melanization and opsonization. Immunobiology 211, 213–236. doi:10.1016/j.imbio.2005.10.015

Kacsoh BZ, Bozler J, Schlenke TA. 2014. A role for nematocytes in the cellular immune response of the drosophilid *Zaprionus indianus.* Parasitology. 2014 Apr;141(5):697-715. doi: 10.1017/S0031182013001431.

Köhler, G., Milstein, C., 1976. Derivation of specific antibody-producing tissue culture and tumor lines by cell fusion. Eur. J. Immunol. 6, 511–519. doi:10.1002/eji.1830060713

Köhler, G., Milstein, C., 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 256, 495–497.

Kotani, E., Yamakawa, M., Iwamoto, S., Tashiro, M., Mori, H., Sumida, M., Matsubara, F., Taniai, K., Kadono-Okuda, K., Kato, Y., 1995. Cloning and expression of the gene of hemocytin, an insect humoral lectin which is homologous with the mammalian von Willebrand factor. Biochim. Biophys. Acta 1260, 245–258.

Kurucz, E., Zettervall, C.-J., Sinka, R., Vilmos, P., Pivarcsi, A., Ekengren, S., Hegedüs, Z., Ando, I., Hultmark, D., 2003. Hemese, a hemocyte-specific transmembrane protein, affects the cellular immune response in *Drosophila*. Proc. Natl. Acad. Sci. U.S.A. 100, 2622–2627. doi:10.1073/pnas.0436940100

Kurucz, E., Márkus, R., Zsámboki, J., Folkl-Medzihradszky, K., Darula, Z., Vilmos, P., Udvardy, A., Krausz, I., Lukacsovich, T., Gateff, E., Zettervall, C.-J., Hultmark, D., Andó, I., 2007a. Nimrod, a putative phagocytosis receptor with EGF repeats in *Drosophila* plasmatocytes. Curr. Biol. 17, 649–654. doi:10.1016/j.cub.2007.02.041

Kurucz, E., Váczi, B., Márkus, R., Laurinyecz, B., Vilmos, P., Zsámboki, J., Csorba, K., Gateff, E., Hultmark, D., Andó, I., 2007b. Definition of *Drosophila* hemocyte subsets by cell-type specific antigens. Acta. Biol. Hung. 58 Suppl, 95–111. doi:10.1556/ABiol.58.2007.Suppl.8

Lanot R, Zachary D, Holder F, Meister M. 2001. Postembryonic hematopoiesis in *Drosophila*. Dev Biol. 2001 Feb 15;230(2):243-57.

Lavine, M.D., Strand, M.R., 2002. Insect hemocytes and their role in immunity. Insect Biochem.Mol. Biol. 32, 1295–1309.

Lesch, C., Goto, A., Lindgren, M., Bidla, G., Dushay, M.S., Theopold, U., 2007. A role for Hemolectin in coagulation and immunity in *Drosophila melanogaster*. Dev. Comp. Immunol. 31, 1255–1263. doi:10.1016/j.dci.2007.03.012

Márkus, R., Laurinyecz, B., Kurucz, E., Honti, V., Bajusz, I., Sipos, B., Somogyi, K., Kronhamn, J., Hultmark, D., Andó, I., 2009. Sessile hemocytes as a hematopoietic compartment in *Drosophila melanogaster*. Proc. Natl. Acad. Sci. U.S.A. 106, 4805–4809. doi:10.1073/pnas.0801766106

Márkus, R., Lerner, Z., Honti, V., Csordás, G., Zsámboki, J., Cinege, G., Párducz, Á., Lukacsovich, T., Kurucz, É., Andó, I., 2015. Multinucleated Giant Hemocytes Are Effector Cells in Cell-Mediated Immune Responses of *Drosophila*. J Innate Immun 7, 340–353. doi:10.1159/000369618

Marringa, W.J., Krueger, M.J., Burritt, N.L., Burritt, J.B., 2014. Honey Bee Hemocyte Profiling by Flow Cytometry. PLoS One 9. doi:10.1371/journal.pone.0108486

Martin, S.J., Medina, L.M., 2004. Africanized honeybees have unique tolerance to *Varroa* mites. Trends in Parasitology 20, 112–114. doi:10.1016/j.pt.2004.01.001

Meister M. 2004. Blood cells of *Drosophila*: cell lineages and role in host defence. Curr Opin Immunol. 16:10-5.

Negri, P., Maggi, M., Ramirez, L., Szawarski, N., Feudis, L.D., Lamattina, L., Eguaras, M., 2015. Cellular immunity in *Apis mellifera*: studying hemocytes brings light about bees skills to confront threats. Apidologie 47, 379–388. doi:10.1007/s13592-015-0418-2

Negri, P., Maggi, M., Szawarski, N., Lamattina, L., Eguaras, M., 2014. *Apis mellifera* haemocytes in-vitro, What type of cells are they? Functional analysis before and after pupal metamorphosis. Journal of Apicultural Research 53, 576–589. doi:10.3896/IBRA.1.53.5.11

Nunes, F.M.F., Simões, Z.L.P., 2009. A non-invasive method for silencing gene transcription in honeybees maintained under natural conditions. Insect Biochem. Mol. Biol. 39, 157–160. doi:10.1016/j.ibmb.2008.10.011

Renault, S., Petit, A., Bénédet, F., Bigot, S., Bigot, Y., 2002. Effects of the Diadromus pulchellus ascovirus, DpAV-4, on the hemocytic encapsulation response and capsule melanization of the leek-moth pupa, *Acrolepiopsis assectella*. J. Insect Physiol. 48, 297–302.

Rizki, M.T.M., 1957. Alterations in the haemocyte population of *Drosophila melanogaster*. J. Morphol. 100, 437–458. doi:10.1002/jmor.1051000303

Rizki TM, Rizki RM. 1992. Lamellocyte differentiation in *Drosophila* larvae parasitized by *Leptopilina*. Dev Comp Immunol. 1992 Mar-Jun;16(2-3):103-10.

Rodriguez, A., Zhou, Z., Tang, M.L., Meller, S., Chen, J., Bellen, H., Kimbrell, D.A., 1996. Identification of immune system and response genes, and novel mutations causing melanotic tumor formation in *Drosophila melanogaster*. Genetics 143, 929–940.

Rus, F., Kurucz, E., Márkus, R., Sinenko, S.A., Laurinyecz, B., Pataki, C., Gausz, J., Hegedűs, Z., Udvardy, A., Hultmark, D., Andó, I., 2006. Expression pattern of Filamin-240 in *Drosophila* blood cells. Gene Expr. Patterns 6, 928–934. doi:10.1016/j.modgep.2006.03.005

Schmid, M.R., Brockmann, A., Pirk, C.W.W., Stanley, D.W., Tautz, J., 2008. Adult honeybees (*Apis mellifera L.*) abandon hemocytic, but not phenoloxidase-based immunity. J. Insect Physiol. 54, 439–444. doi:10.1016/j.jinsphys.2007.11.002

Simon-Delso, N., San Martin, G., Bruneau, E., Minsart, L.-A., Mouret, C., Hautier, L., 2014. Honeybee colony disorder in crop areas: the role of pesticides and viruses. PLoS ONE 9, e103073. doi:10.1371/journal.pone.0103073

Theopold, U., Li, D., Fabbri, M., Scherfer, C., Schmidt, O.,2002. The coagulation of insect hemolymph. Cell Mol. Life Sci. 59, 363–672.

vanEngelsdorp, D., Evans, J.D., Saegerman, C., Mullin, C., Haubruge, E., Nguyen, B.K., Frazier, M., Frazier, J., Cox-Foster, D., Chen, Y., Underwood, R., Tarpy, D.R., Pettis, J.S., 2009. Colony Collapse Disorder: A Descriptive Study. PLoS ONE 4, e6481. doi:10.1371/journal.pone.0006481

Van Steenkiste D. 1988. De hemocyten van de honingbij (*Apis mellifera L.*): typologie, bloedbeeld en cellulaire verdedigingsreacties, Ph.D.-thesis, University of Ghent.

Vilmos, P., Nagy, I., Kurucz, E., Hultmark, D., Gateff, E., Andó, I., 2004. A rapid rosetting method for separation of hemocyte sub-populations of *Drosophila melanogaster*. Dev. Comp. Immunol. 28, 555–563. doi:10.1016/j.dci.2003.10.001

Wallberg, A., Han, F., Wellhagen, G., Dahle, B., Kawata, M., Haddad, N., Simões, Z.L.P., Allsopp, M.H., Kandemir, I., De la Rúa, P., Pirk, C.W., Webster, M.T., 2014. A worldwide survey of genome sequence variation provides insight into the evolutionary history of the honeybee *Apis mellifera*. Nat Genet 46, 1081–1088. doi:10.1038/ng.3077

Williams, M.J., Ando, I., Hultmark, D., 2005. *Drosophila melanogaster Rac2* is necessary for a proper cellular immune response. Genes Cells 10, 813–823. doi:10.1111/j.1365-2443.2005.00883.x

Williams, M.J., 2007. *Drosophila* hemopoiesis and cellular immunity. J. Immunol. 178, 4711–4716.

Zettervall, C.-J., Anderl, I., Williams, M.J., Palmer, R., Kurucz, E., Ando, I., Hultmark, D., 2004. A directed screen for genes involved in *Drosophila* blood cell activation. Proc. Natl. Acad. Sci. U.S.A. 101, 14192–14197. doi:10.1073/pnas.0403789101

Appendix Supplementary data

Supplementary Material

LC-MS/MS analysis

The sample was treated according to the general in-gel digestion protocol (http://ms-facility.ucsf.edu/ingel.html). Briefly, after silver staining was removed using Farmer's reducer; salts, and SDS were washed out, disulfide bridges were reduced with dithiothreitol, and the resulting free sulfhydryls were alkylated with iodoacetamide. Digestion with side-chain protected porcine trypsin (Promega) proceeded at 37 °C for 4 h. The resulting peptides were extracted from the gel using 2% formic acid in 50% acetonitrile, then the samples were dried down and dissolved in 0.1% formic acid. MS data were acquired on an LTQ Orbitrap-Elite mass spectrometer (Thermo Scientific) on-line coupled to a Waters nanoAcquity HPLC. Samples were injected onto a trapping column (home-made 0.18 mm ID x 52 mm length column, packed with Magic C18 AQ (3µm particle size, 200Å pore size)) with 3% solvent B at 3 µl/min then were transferred onto the separating column (home-made C18, 0.075 mm ID x 83 mm length column packed with Magic C18 AQ (3µm particle size, 200Å pore size)) using a linear gradient of 3-40% solvent B in 37 min at 500 nl/min. Solvent A: 0.1% formic acid / water, solvent B: 0.1% formic acid / 5% dimethyl sulfoxide / acetonitrile. MS data acquisition was carried out in a data-dependent fashion acquiring CID spectra of the 10 most intense, multiply charged precursor ions identified from each MS survey scan. MS spectra were acquired in the Orbitrap, and CID spectra in the linear ion trap. Dynamic exclusion was enabled (exclusion time: 30 s, mass width: ±10ppm).

Raw data were converted into peak-lists using the PAVA software [A data processing pipeline for mammalian proteome dynamics studies using stable isotope metabolic labeling. Guan S, Price JC, Prusiner SB, Ghaemmaghami S, Burlingame AL. Mol Cell Proteomics. 2011 Dec;10(12):M111.010728.] and searched with the Protein Prospector search engine (v.5.10.17.). In order to identify probable protein contamination, a database search using the Swissprot database (2014.03.07.version, supplemented with a random sequence for each entry; 542503 entries searched) was performed first; then another search was performed using the Uniprot database with *Apis mellifera* and *Bos taurus* species restriction (2014.03.12.version, supplemented with a random sequence for each entry; 44093 entries searched) with additional proteins identified from the Swissprot search (such as human

keratins) included. Additional search parameters were the same in both searches: enzyme: trypsin with maximum 1 missed cleavage site; fixed modifications: carbamidomethyl (Cys); variable modifications: acetyl (protein N-terminus), Gln->pyro-Glu (peptide N-terminal Gln),oxidation (Met); mass accuracy: ±5 ppm for precursors and ±0.6 Da for fragment ions (both defined as monoisotopic m/z values); instrument type: ESI-TRAP. As acceptance criteria we used the default settings, i.e. max E-values were 0.01 and 0.05 for proteins and peptides, respectively.

Sequence coverage (Suppl. Table 1.) and list of peptides identified (Suppl. Table 2.) for Uniprot ID: A0A087ZSR0 are listed in Supplementary Tables. Hml peptides are highlighted with bold.

1	MLWKIFFNIA	IIISIFNQFV	IGDNFLKSSD	ITETTISLND	ISKLNYEDMK	NVKSKTKQLL	FPGGCSK**QPD**	**SPINGEIR**CS
81	IDSGCIATCK	HDYKFPNGVT	QLAITCMNEE	WYIHGTDWIS	IPHCEPICLP	ECLNNGVCIA	PHQCNCPEDF	TGPQCQFEKK
161	PCLNYLSPVL	NAHKTCNSQS	CTISCLKNFS	FPDGTSVTNL	LCKNGNWEPT	RKDWVSIPDC	EPICEPPCQN	GGNCLPSNLC
241	QCPQAYRGSQ	CQYSADICNG	EKMGFNGGFF	CSNIDDTYSC	TINCPAGVEF	EFPPASVYIC	NYETGVFMPQ	PIPQCNYSEN
321	MNIISLGTIY	NSYIKETNHT	WTYQDIFNPH	TNQFPLIHGN	YGIKEHYSNH	KSNITTNTMI	FNPLENNMLF	IEEKKPTPKT
401	CFTWNGVHYK	TFDDGIFTFD	SECSYILVQE	AQNRLFTVTV	NNSPTCEVQD	CFKVIKIYIQ	DKEYILSRNK	EGVPEFRTRK
481	KLLPIPAQLS	TLRVEMSAHF	IVVILDSLGI	QLKWDGALML	QVEAAENMWN	KTIGLCGNMN	GDKSDDLISK	NGKYTK**SVAS**
561	**FATSWK**TEDI	GETCDKYPII	KHSCESDSLI	TKDAIQFCAK	LFSDYRFKAC	SNTISVSELQ	IACLWDYCSC	EDYDRRKCAC
641	NTMNVYIRQC	AHKKIISVSG	WRNNDTCPMT	CNGGRVYMPC	GPKIESSCWT	EKELNIENCE	EGCFCPEGTV	AHEGKCIYPN
721	ECPCRLRGKL	FQPGKIVQKD	CNTCTCSSGK	WICTQLKCSA	RCAVIGDPHY	VTFDGKHYDF	MGKCKYYLMK	DDDYSIEGEN
801	VPCSGAISEN	MGLIPSNAPS	CTKTVTINYK	DTSMKLKQHR	QVLINGNELT	IFPTLINGIR	IRIASSIFLI	VQLPNGLEIW
881	WDGISR**IYIN**	**APPEFHGNTK**	GLCGTFSENQ	KDDFITPEGD	IENTAISFAN	KWKCDEVCPN	VPEKELDHPC	DLNPQKRVTA
961	KQYCSYLFSN	IFTDCHWYVD	PDTFYKDCLF	DMCSCKVELE	SCLCPILAAY	AKDCSTAGIK	LLWRQNVEEC	KIHCSGSQVY
1041	QICGNSCTRS	CGDISFYQNC	KQDCVEGCNC	PEGETLDIHG	ECIPIGQCPC	TYGGLEFSSG	HKEIR**SENKF**	**PELCTCAGGI**
1121	**WNCR**EAMPNE	IIKYPAVKNL	LTSCLISNHQ	EITDCIQTEP	RTCYNMHKPI	QKPSICKSGC	VCKSGYVLNE	PNGNCIKEET
1201	CPCHHGSR**SY**	**EEESIIQNEC**	**NTCK**CTNGTW	KCTDRICAGI	CSVWGDSHYK	TFDGKIYDFQ	GLCDYILVKG	SLSQEDCFDI
1281	SIQNVPCGTT	GVSCSK**SITL**	**TIGSDQSSER**	IVLTRGKTLP	LDNFKRIIMR	TAGLFLFINV	PDIGLTMQWD	KGTRVYVRLE
1361	PKWKGRTKGL	CGDYNNNSED	DFK**TPSGGIS**	**EVSANLFGDS**	**WK**KNEFCPEP	KNIQDPCVQH	PERKLWATQK	CGILKSSIFQ
1441	SCHSEVEVES	YIHNCIFDSC	SCDTGGDCEC	LCTALAAYAQ	ECNAKGIPIK	WRSQELCPIQ	CDENCSSYSP	CITTCPQETC
1521	DNLIILNDKS	HLCSQDICVE	GCSIKSCPKN	QVYSNNSYTE	CVPKEICKTP	CTEINGITYY	EGDNVNNDDC	RTCYCSRGKV
1601	LCKGEPCTNI	IMPSTVPLEE	PQKCVNGWTN	WINQDPAIKG	KK**FKDIEPLP**	**STLILSNIK**G	SAICDKNQMI	DIKCR**SVNDH**

1681	**LTPKETGLDV**	**ECSLER**GLYC	QSHFNLSCID	FEISVLCQCP	SITTEK**LDIS**	**TATETNLFGK**	CNIEFQNEPH	PTDCHLFYQC
1761	IPGINGNEFI	KKSCEENMLY	NPQTQVCDWP	ATVILIRPEC	SMKQITPNKI	EWTSDKKIKY	KTTTSTTLEK	NIIISKTCKE
1841	NEIWNDCAIN	CNKVCDYYKY	ILLKEGKCNG	ISDCIAGCVS	LEKPQCQPNE	FWRDAMTCVN	EDDCSCRSHN	GHPVISGAIL
1921	MESECEICQC	IKNYYTCDKS	SCFTEINNMT	TEQPNMQSST	ETISSLFEIH	TFIVPSTVSP	PSYCISNNFV	PLIQYLDNQV
2001	SFDASTIKDS	NFQSKNALLK	KIGFWEPEYD	TTDQWLDIKF	QK**SEPIYGII**	**IQGNTIENK**F	VTSYRILFSE	DAHLFSYVMD
2081	NKKKPQIFRG	PMDQFKLVEQ	KFYQPIEARI	VRINPLSWHN	GIAMKIELLG	CQEMVSTVIP	VTETIPIITT	TITEKIIVPM
2161	CDEPMGLDDG	IIFSDQILVS	SSSTDLLPNL	KLSSPKIWHP	KLHNPHQFVK	**IDFLEPR**NLT	GIATKGGEGT	WTTVYKVFYS
2241	NNDYQWNPVM	DDNGNEREFL	GNFDSNTIKK	NYFDKPLNAR	YLKIQPIKWH	EQIGLKFEVF	GCFLPYPLKT	TTEKLEITSM
2321	TTQTFEKCNV	CEGIQNEDQI	TCKCKESLWW	NGNTCVIKQE	CPCVVEHILY	NVGAIYVNKE	CQECICTLGG	TSFCHPKKCK
2401	PCQELGKRPV	VNELCNCICK	SCPSGTRHCP	TSDVCIDDNL	WCNGIQDCPD	DEKDCPNITT	KPEEITTMKI	ESTTITSTSD
2481	IPIVCQDPIC	PPEYKIILKR	PQNSLYYTKS	YVKEGIKSLH	TKLSRRKGLR	KSMQYHIKYT	DENEKSKNEI	ECTQFTCVPI
2561	KPWTIFNENV	SQSCPKIFCP	PDYTIIYEKI	SMYKHQKCPK	YSCKPPTPKE	VICNITGRTF	NTFDKLEYKY	DICNHILARD
2641	MFANTWYITM	EKQCDLHMGQ	CTKILVVTLD	EDVILLYPDM	HIDINEYTFT	SNQINRIDNK	FHSFKMSTIG	NVIYLVSNYY
2721	GFWIIWDINS	NVKIGISTKL	IHQVDGLCGY	FDGYSINDK**Q**	**LPDGSQAR**ST	VEFGNSWVIE	GTPECDPQIC	PYQLQAKSWD
2801	ICNIVKDASL	TECSNIVNLE	KFISSCMENI	CNCLHSNHSY	DDCRCRLLTS	FVTECQAGDL	NIDLSTWRSI	HDCPVICASP
2881	LVHKDCFRNK	CETSCNNLQQ	IDPCPIMQGV	CFSGCFCPDG	TVRNGDECVP	PTYCKDCICE	WLGNSKFISF	DRKNIKFDGN
2961	CTYILSRNIV	ENVKRNKKYT	YQILVSNKIC	DTGTCTEMII	ILYQNHVIKI	KESIPNQEFE	IEVDDSKIYE	LPFNKSWLIL
3041	KHTSLKKLRL	LIPSIQLEII	GYQPNFAFSL	SVPSHIFGGA	VEGLCGNCNE	DPEDDLKQQD	GKVTKDIQDF	ITSWLVTESP
3121	NINLNTNICV	FNNQSKCISP	DQDLCQKLLN	IADFGLCHNL	VDPMPYFMAC	KDNMCSGGSY	CNSFEAYSRK	CQQMGVCLTW
3201	RSSKICPYIC	PSHLVYQPCN	STCKQTCDMI	NEMNDMCIKN	YEEGCFCPQN	LIFHNGTCIS	KEKCLLCDEE	GHIEGDIWFL
3281	DICTKCTCNK	KTVKCEKTEC	PAVETICEEN	MTPMIINGTE	KDCCVKYLCI	PKTVTTMTPF	CIEPQIPECG	YGQIIKAFVD

3361	SDGCKKFICE	CVPSSECPIL	NEISLEVDQL	QPGFKQVTNT	SGCCPKFMTI	CDPQTCPSAP	SCPEYHELKI	DTKNACCNIY
3441	KCDPPKDLCL	YNIEFESKIE	MTEHIVAKKL	GEQWMDGKYD	KCATEICALE	PNITKYKEIE	VCNKNCTPGW	IYEEKENECC
3521	GQCKQAYCII	EDMFYKPNTT	WYSIDNCTIF	TCIKQGEQLV	ISSSSVVCPD	VTDCPDTLLY	MQNCCKICNL	TSYNHKIESC
3601	VANVLEEQNT	IGMFSIKHRV	HGLCKNLEPI	NGITECHGIC	ESNSYFDTDN	WSQIVNCQCC	QPTEYKSLIV	ELICEDNKKF
3681	EKQVTVPVSC	ACSTCMSNEK	IYKRRKDGVK	G				

Supplementary Table 1. Sequence coverage identified for Uniprot ID: A0A087ZSR0

1. Acc. #: A0A087ZSR0 Uniprot ID: A0A087ZSR0_APIME Species: APIME Name: Uncharacterized protein
Organism: *Apis mellifera* Existence: Predicted Version: 1
Protein MW: 420327.5 Protein pI: 5.4 Protein Length: 3711

Search Name	Num Unique	Peptide Count	% Cov
141114_02_KuruczE_3_ITMSms2cid/UP restr bee bovine	14	17	5.1

m/z	z	ppm	Peptide	M Cl	RT	Score	Expect
613.3140	2	2.9	**(K)QPDSPINGEIR**	0	30.823	18.5	1.6e-4
542.2783	2	2.2	**(K)SVASFATSWK**	0	38.020	23.0	4.1e-4
534.2767	3	2.3	**(R)IYINAPPEFHGNTK**	0	34.391	17.0	0.0061
767.0068	3	0.25	**(R)SENKFPELCTCAGGIWNCR**	1	39.890	19.0	2.6e-4
1002.4245	2	4.8	**(R)SYEEESIIQNECNTCK**	0	32.947	30.5	3.8e-7
747.3776	2	2.5	**(K)SITLTIGSDQSSER**	0	34.527	22.9	9.7e-5
747.3765	2	1.0	**(K)SITLTIGSDQSSER**	0	35.075	30.0	3.0e-5
976.4713	2	-2.2	**(K)TPSGGISEVSANLFGDSWK**	0	44.337	29.9	8.3e-7
643.3750	3	0.081	**(K)FKDIEPLPSTLILSNIK**	1	44.097	23.6	7.6e-5
505.7678	2	1.8	**(R)SVNDHLTPK**	0	23.610	19.6	0.0017
505.7672	2	0.58	**(R)SVNDHLTPK**	0	24.124	24.8	1.4e-4
704.3274	2	3.9	**(K)ETGLDVECSLER**	0	34.953	16.1	0.0021
755.3932	2	-0.26	**(K)LDISTATETNLFGK**	0	40.205	25.7	2.8e-5

945.0068	2	2.6	**(K)SEPIYGIIIQGNTIENK**	0	41.673	40.6	2.2e-7
445.2434	2	1.9	**(K)IDFLEPR**	0	37.251	20.9	0.0019
445.2430	2	1.0	**(K)IDFLEPR**	0	37.762	23.1	4.5e-4
486.2491	2	0.45	**(K)QLPDGSQAR**	0	24.005	22.9	0.0018

Supplementary Table 2. List of peptides identified for Uniprot ID: A0A087ZSR0