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RESEARCH ARTICLE

Honey bee drones maintain humoral immune competence throughout all life stages in the absence of vitellogenin production

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SUMMARY

Drones are haploid male individuals whose major social function in honey bee colonies is to produce sperm and mate with a queen. In spite of their limited tasks, the vitality of drones is of utmost importance for the next generation. The immune competence of drones – as compared to worker bees – is largely unexplored. Hence, we studied humoral and cellular immune reactions of *in vitro* reared drone larvae and adult drones of different age upon artificial bacterial infection. Haemolymph samples were collected after aseptic and septic injury and subsequently employed for (1) the identification of immune-responsive peptides and/or proteins by qualitative proteomic analyses in combination with mass spectrometry and (2) the detection of antimicrobial activity by inhibition-zone assays. Drone larvae and adult drones responded with a strong humoral immune reaction upon bacterial challenge, as validated by the expression of small antimicrobial peptides. Young adult drones exhibited a broader spectrum of defence reactions than drone larvae. Distinct polypeptides including peptidoglycan recognition protein-S2 and lysozyme 2 were upregulated in immunized adult drones. Moreover, a pronounced nodulation reaction was observed in young drones upon bacterial challenge. Prophenoloxidase zymogen is present at an almost constant level in non-infected adult drones throughout the entire lifespan. All observed immune reactions in drones were expressed in the absence of significant amounts of vitellogenin. We conclude that drones – like worker bees – have the potential to activate multiple elements of the innate immune response.

Supplementary material available online at http://jeb.biologists.org/cgi/content/full/215/8/1313/DC1

Key words: cellular defence, drone, honey bee, insect immunity, lysozyme, nodulation, phenoloxidase, vitellogenin.

INTRODUCTION

Honey bees – like all insects – lack an adaptive immune system. Instead they rely on innate immune reactions to combat intruding microorganisms. The innate immune response is manifested in three major ways: a constitutively active cellular system that includes phagocytosis and nodulation formation, an induced humoral immune response that ultimately generates small antimicrobial peptides (AMPs) and the activation of prophenoloxidase (proPO) by a serine protease cascade resulting in the transient synthesis of quinones and melanin (Hultmark, 2003; Lemaitre and Hofmann, 2007; Cerenius et al., 2008). Two distinct signalling pathways, referred to as Toll and Imd pathways, play key roles in the regulation of transcription of AMP genes. These pathways are usually activated by determinants that are conserved in the cell wall of microbes but absent in the host, such as lipopolysaccharides (LPSs) or peptidoglycans (Lemaitre and Hoffmann, 2007). The recent sequencing of the honey bee genome (Honey Bee Genome Sequencing Consortium, 2006) and comparison with genomic data from other insects, e.g. *Drosophila melanogaster* and *Anopheles gambiae*, revealed that honey bees possess homologous members of the humoral immune response (Evans et al., 2006). Moreover, a number of AMPs have been identified in honey bees. Casteels and colleagues analysed haemolymph samples collected from young adult worker bees challenged with *Escherichia coli*. Components present in immunized samples but absent in control samples were further purified and subsequently characterized by amino acid sequence analyses. Four different types of AMPs were identified: apidaecins (Casteels et al., 1989), abaecin (Casteels et al., 1990), hymenoptaecin (Casteels et al., 1993) and defensin 1 (Casteels-Josson et al., 1994). Later on, a gene coding for a second defensin (i.e. defensin 2) was detected by genome analysis (Klaudiny et al., 2005).

We have recently studied the defence reactions of honey bee worker larvae and adults and have found that worker larvae – like adult bees – respond with a strong humoral reaction upon artificial bacterial challenge manifested by the production of bee-specific AMPs. In addition, newly emerged adults – but not larvae – reacted with the upregulation of several large proteins, including a member of the carboxylesterase family (CE 1), peptidoglycan recognition protein-S2 (PGRP-S2) and a protein with a molecular mass of 30 kDa (IRp30) of unknown function (Randolt et al., 2008).

Like other eusocial insects, honey bees harbour three castes within a colony: a single queen (fertile female), up to 50,000 workers (sterile females) and approximately 2000 drones (males). Drones do not perform typical worker bee tasks inside and outside the hive such as brood nursing, nest construction or collection of nectar. Instead, their main social function is to produce sperm and mate with a queen (Hrassnigg and Crailsheim, 2005). In temperate regions, drones are found in honey bee colonies only during part of the summer season and their availability for studies is therefore limited to a short period of the year. Despite their restricted functions, drones are important for the maintenance of the next generation. Hence, their overall

vitality together with their capability to cope with microbial infections is crucial during their short lifespan.

Very little is known about the immune competence of drones at each life stage. We studied how drone larvae and drone adults of different age respond to artificial challenge with bacteria. Here, we show that (1) drones react with a similar spectrum of immune reactions as honey bee workers and (2) all relevant activities are expressed in the absence of significant amounts of vitellogenin.

MATERIALS AND METHODS

Bacterial strains and media

The Gram-negative bacterium *Escherichia coli* (DSM 682) was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Braunschweig, Germany) and the Gram-positive bacterium *Micrococcus flavus* was a gift from Dr U. Rdest (Institute of Microbiology, Biocentre, Würzburg, Germany). The *E. coli* strain was cultivated in nutrient broth (NB medium), whereas *M. flavus* was grown in lysogeny broth (LB medium) as described previously (Randolt et al., 2008). All ingredients were purchased from Becton Dickinson (Heidelberg, Germany). For infection experiments, *E. coli* bacteria were grown to an absorbance of A_{550}=0.5 (~3×10^8 cells ml^{-1}). After centrifugation, cells were washed two times and resuspended in phosphate-buffered saline (PBS) at the desired concentration.

In vitro rearing of drone larvae and septic wounding

Drone combs were removed from colonies of *Apis mellifera carnica* Pollman 1879 maintained at the apiary of the BEEstation (University of Würzburg, Germany). Newly hatched drone larvae were collected from the comb with a Swiss grafting tool (Bienen Meier, Künten, Switzerland) and transferred to a 24-well tissue culture plate (No. 662160, Greiner, Frickenhausen, Germany). The basic diet was prepared according to Peng et al. (Peng et al., 1992). It consisted of 4.2 g freeze-dried royal jelly powder (Werner Seip, Butzbach, Germany), 0.6 g fructose, 0.6 g glucose, 0.2 g Difco yeast extract and 14.4 ml sterile deionized water. The wells were filled with 300 µl of this basic diet. On the first day of culture, 10 larvae were placed together in a single well. During the following days, the number of larvae cultured together was stepwise reduced according to their size. The grafted larvae were maintained in an incubator (Memmert, HCP 108, Schwabach, Germany) at 35°C and 70% relative humidity. Each day they were transferred to new tissue culture plates filled with fresh prewarmed diet. After the fifth instar stage, the basic diet was replaced by a modified diet consisting of 3.4 g royal jelly powder, 0.6 g fructose, 1.4 g glucose and 12.6 ml deionized water.

Aseptic and septic wounding of drone larvae was performed in a manner similar to that established for worker larvae (Randolt et al., 2008). For injection, we used disposable calibrated (1–5 µl) glass capillaries (Servoprax, Hartenstein, Würzburg, Germany) with fine tips that were generated by a P-2000 laser based micropipette puller (Sutter Instrument, Novato, CA, USA). Groups of 10 larvae were injected dorsally with either 1 µl PBS or 1 µl of 10^4 viable *E. coli* cells.

In vitro challenge of adult drones with bacteria

Newly emerged drones (up to 24 h old) were collected from a caged drone comb placed in an incubator at 35°C and 65% relative humidity shortly before the first drones emerged from sealed cells. For each series of experiments, the young drones were divided into three groups of 10 to 20 individuals, one of which was kept as an untreated control group [not infected (n.i.)]. The remaining drones were injected laterally between the second and the third tergite into the haemocoel with volumes of 1 µl of buffer (PBS) or bacteria (10^5 viable *E. coli* cells). The drones of each group were kept in small cages at 30°C together with an equal number of worker bees until haemolymph collection. They were supplied with 50% (v/v) ApiInvert (Südzucker, Mannheim, Germany) *ad libitum*.

For longevity experiments, newly emerged drones collected from a caged drone comb (see above) were labeled individually onto the thorax with a paint marker (Edding 750) to indicate the day of emergence and were then reintroduced into the original colony.

Haemolymph collection

At indicated times after septic and aseptic wounding, larvae were bled by puncturing with a fine-tipped glass capillary (see above). The haemolymph of drones was collected *via* a leg wound with a glass capillary and transferred to reaction tubes containing mixtures of N-phenylthiourea (PTU) and aprotinin as described previously (Randolt et al., 2008). The samples were kept at –20°C until further analyses.

Inhibition-zone assay

An aliquot (0.2 ml) of a fresh overnight bacterial culture was spread onto agar plates (Ø=9 cm) containing NB or LB medium. The test bacteria were either *E. coli 682* (Gram-negative) or *M. flavus* (Gram-positive). As soon as the bacterial layer had been adsorbed, 1.5 µl of undiluted haemolymph samples were applied as a droplet onto the plates with a pipette tip. After 24 h of incubation at 37°C, the clear zones of inhibition were documented by photography against a dark background.

Assay for nodulation reactions

Nodule formation was assessed in newly emerged adult drones and in drone larvae of the seventh, eighth, ninth and tenth instar stage at selected times (1 to 24 h) after injections of 10^5 viable *E. coli* cells into the haemocoel. Before analysis, the drones were embedded in paraffin (Histosec, Merck, Darmstadt, Germany). The dorsal abdominal tergits were carefully removed and the melanised, darkened nodules were counted under a stereomicroscope (Olympus SZX7, Hamburg, Germany).

One-dimensional gel electrophoresis

One-dimensional gel electrophoresis was carried out in vertical polyacrylamide slab gels (13×8.5×0.1 cm) containing 0.1% sodium dodecyl sulfate (SDS) with a 5% stacking gel on top of the separation gel (Laemmli, 1970). Haemolymph samples (1–1.5 µl) were diluted with twofold concentrated sample buffer, heated for 3 min at 95°C and subjected to electrophoresis at constant voltage (130 V). As a rule, 10 and 15% polyacrylamide gels were run in parallel with the same haemolymph samples. After electrophoresis, gels were first fixed for 30 min in 0.85% o-phosphoric acid/20% methanol followed by colloidal Coomassie Blue G250 staining overnight in a solution of Roti®-Blue (Roth, Karlsruhe, Germany) and 20% methanol. Gels were destained in 25% methanol.

Two-dimensional gel electrophoresis

Two-dimensional gel electrophoresis was carried out using precast immobilized pH gradient (IPG) gel strips in an Ettan™IPGphor™3 isoelectric focusing system (GE Healthcare, München, Germany) in the first dimension, followed by the second dimension in vertical polyacrylamide/SDS slab gels. Haemolymph was collected from two individuals (for each single analysis) and immediately centrifuged (10,000 ***g***, 7 min, 4°C). An aliquot (7–8 µl) of the pooled haemolymph containing 125 µg total soluble protein was added to DeStreak

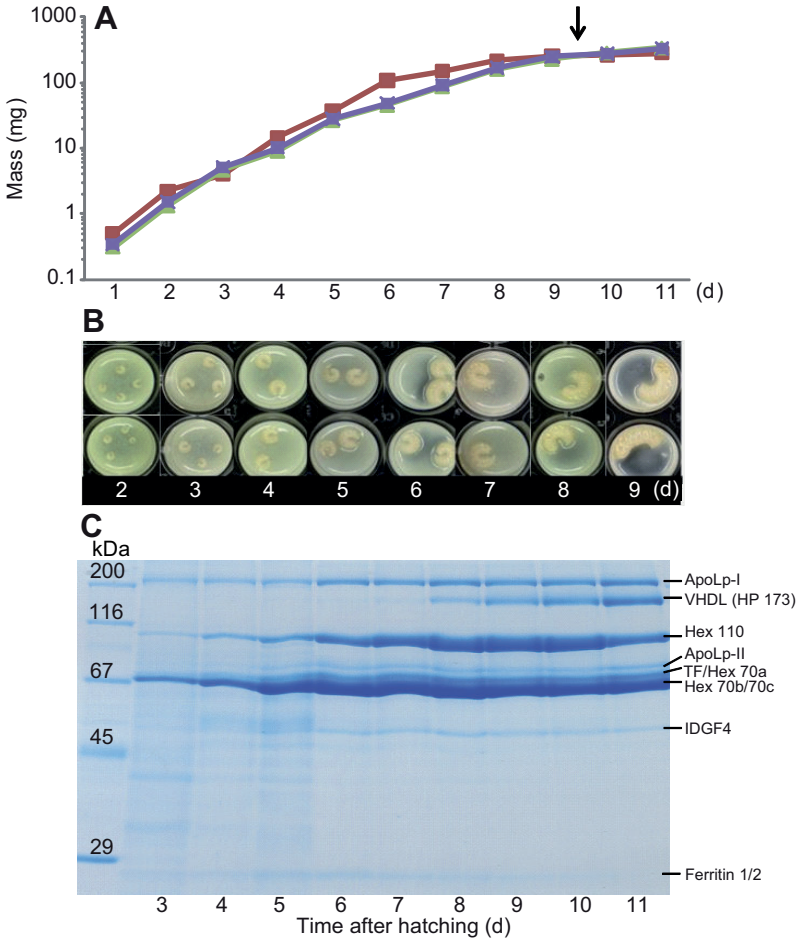

Fig. 1. Development of *in vitro* reared *Apis mellifera carnica* drone larvae. (A) Growth rates of drone larvae. Larvae were collected from brood combs and transferred to tissue culture plates supplemented with a mixed diet. A subset of larvae was weighed each day upon cultivation. To evaluate the average mass of drone larvae, a total of 20 (at the age of 1 and 2 days), five (3–7 days) and three individuals (8–11 days) were combined. Growth rates were determined in three independent series of experiments: series I, carried out in May 2008 (red squares); series II, carried out in May 2010 (green triangles) and series III, carried out in June 2010 (purple squares). (B) Images of *in vitro* reared drone larvae between 2 and 9 days after hatching. The cultivated drone larvae shown here originate from series III. The arrow in A indicates the onset of the prepupation stage. (C) Gel electrophoretic analyses of major haemolymph proteins expressed in drone larvae at different developmental stages. Haemolymph samples were collected from five individual larvae each day upon cultivation (series II) followed by gel analysis for each replicate. An aliquot of these samples (1 μl) was mixed with dissociation buffer and applied onto a 10% polyacrylamide/0.1% SDS gel. A representative haemolymph sample of each larval group is shown. The identification of gel-excised proteins was by MS/MS analyses: ApoLp-I and ApoLp-II, apolipophorin (gil66513966); HP 173=VHDL, very high-density lipoprotein (gil110762106); Hex 110, hexamerin 110 (gil155369750); Hex 70a, hexamerin 70a (gil148357839); Hex 70b, hexamerin 70b (gil58585148); Hex 70c, hexamerin 70c (gil148887477); TF, transferrin (gil58585086); IDGF 4, imaginal disc growth factor 4 (gil66514614); ferritin 1 (gil110762641); ferritin 2 (gil66524161).

rehydration solution (GE Healthcare) and 0.5% immobilized pH gradient (IPG) buffer 3-11 NL (GE Healthcare) for a final volume of 340 μl. The mixture was transferred into an 18 cm strip holder tray (GE Healthcare) and then an IPGphor gel strip (pH 3–11, 18 cm, GE Healthcare) was carefully placed over the protein sample. IPG gel strips were actively rehydrated for 12 h at 20°C and 30 V. Isoelectric focusing (IEF) was carried out at 500 V for 1 h, at 1000 V (gradient) for 1 h, at 8000 V (gradient) for 3 h and at 8000 V for 1.5 h. Prior to SDS-PAGE, the IPG strips were first incubated for 15 min in equilibration buffer (EB) containing 6 mol l^{-1} urea, 75 mmol l^{-1} Tris-HCl, pH 8.8, 29% glycerol, 2% SDS and 0.002% Bromophenol Blue plus 1% dithiothreitol followed by incubation in the same EB supplemented with 2.5% iodoacetamide. The IEF strip was then placed on top of a lab-cast 15% polyacrylamide/0.1% SDS gel (20×18×0.1 cm) and covered with agarose sealing solution (25 mmol l^{-1} Tris base, 192 mmol l^{-1} glycine, 0.1% SDS, 0.5% agarose and 0.002% Bromophenol Blue). Electrophoresis was performed for 45 min at 60 V, followed by a constant 160 V until the Bromophenol Blue dye had reached the bottom of the gel. Gels were stained and destained as described above.

Mass spectrometry analysis and protein identification

Stained protein bands or spots were excised from the gel and subjected to in-gel digestion with trypsin. Protein identification by nano liquid chromatography, in conjunction with electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) analysis and an NCBInr protein database search, was performed by Proteome Factory AG (Berlin, Germany).

Western blotting

For immunoblots, size-separated proteins were electrophoretically transferred to a polyvinylidine fluoride (PVDF) membrane (Immobilon P transfer membrane, Millipore) using a Bio-Rad Transblot apparatus (Bio-Rad Laboratories, Hercules, CA, USA). Membranes were incubated overnight in blocking solution [5% skim milk in Tris-buffered saline, 0.05% Tween 20 (TBST)]. Subsequently, the blots were probed with an antibody raised against the prophenoloxidase peptide sequence C-QPGKNTIEQKSTKS-NH_2 (GenScript, Piscataway, NJ, USA) for 1 h at room temperature at a dilution of 1:1000. The blots were washed three times in TBST followed by incubation with anti-rabbit IgG horseradish peroxidase-conjugated antibodies (Sigma, A6154) for 30 min at a dilution of 1:5000. The detection of immunoreactive proteins was carried out with the enhanced chemiluminescence kit (GE Healthcare).

RESULTS

Development of *in vitro* reared drone larvae

We have recently established the *in vitro* cultivation of worker bee larvae (Randolt et al., 2008) using as a basic diet a mixture of freeze-dried royal jelly, fructose, glucose and yeast extract (Peng et al., 1992). Drone larvae developed rapidly in this diet within the first 5 days after hatching, but later on the development was impaired and larval mortality was relatively high, i.e. up to 20% of drone larvae did not survive. Hence, between 6 and 10 days after hatching we switched to a modified diet that contained a reduced amount of royal jelly, no yeast extract and an increased supply of glucose. Under these conditions, up to 93% of *in vitro* reared drone larvae

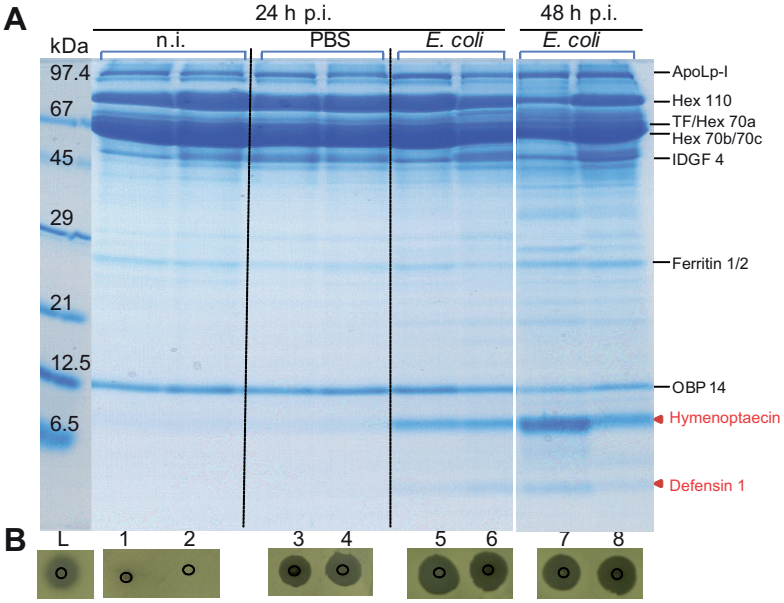

Fig. 2. Characterization of induced antimicrobial peptides (AMPs) in honey bee drone larvae. (A) Gel electrophoretic analysis of haemolymph proteins. Six-day-old larvae were left untreated (n.i.), mock-infected with buffer (PBS) or challenged with 10^4 viable *Escherichia coli* cells. Haemolymph samples were collected 24 and 48 h post-injection (p.i.) followed by gel analysis of two replicates. An aliquot of these samples (1 µl) was mixed with dissociation buffer and applied onto a 15% polyacrylamide/0.1% SDS gel. Gels were stained with Coomassie Brilliant Blue G250. AMPs are indicated by red arrowheads. (B) Aliquots of the same haemolymph samples were applied on agar plates together with *Micrococcus flavus* as indicator bacteria. As a positive control, lysozyme (L) was employed at a concentration of 5 µg µl^{-1}.

survived until the prepupal stage. Moreover, drone larval development was rather reproducible as documented by three independent growth curves (Fig. 1A) and it proceeded similar to that of drone larval brood reared in the hive (Fig. 1B) (Winston, 1987). Until 6 days after hatching, larval mass increased exponentially and culminated in an average mass of 250 mg larva^{-1} of 9-day-old drone larvae. *In vitro* rearing of honey bee drones has also been described by Behrens et al. (Behrens et al., 2007), who employed a slightly different diet.

Drone larval development was also studied using qualitative protein analyses of haemolymph proteins at each larval stage (Fig. 1C) (supplementary material Table S1). Between 5 and 11 days after hatching, three hexamerins in the 70 kDa range (hexamerins 70a, 70b and 70c) and one hexamerin of 110 kDa comprise the bulk of haemolymph proteins. These proteins have evolved from copper-containing haemocyanins and serve as storage proteins in insects, i.e. as source of amino acids for tissue reconstruction during pupal development (Danty et al., 1998; Burmester and Scheller, 1999; Martins et al., 2010). At late larval stages, another prominent protein was present in haemolymph samples. It is annotated as 'hypothetical protein' with a predicted mass of 173 kDa in the NCBI database. However, it has been isolated previously from larval haemolymph and assigned as 'very high density lipoprotein' (VHDL) by Shipman et al. (Shipman et al., 1987). Determination of the N-terminal amino acid sequence of the secreted VHDL by Edman degradation carried out by this group (Shipman et al., 1987) revealed its relationship to HP 173. Throughout the total larval development, imaginal disc growth factor 4 (IDGF 4) and ferritins 1 and 2 are available at rather constant levels. IDGFs are a family of growth factors related to chitinase enzymes that are required by *Drosophila* imaginal disc cells (Kawamura et al., 1999). Ferritins belong to a class of polymeric iron-binding proteins that sequester potentially toxic free iron and that are mainly involved in iron storage (Dunkov and Georgieva, 2006).

Wounding and septic injury induces a strong humoral reaction in drone larvae

Drone larvae were challenged with different elicitors at different developmental stages. Viable *E. coli* cells induced the synthesis of the two AMPs hymenoptaecin and defensin 1 in 6-day-old drone larvae (Fig. 2A, supplementary material Table S2). A similar response was observed upon bacterial challenge of 5-, 7- and 8-day-old drone larvae (data not shown). The presence of AMPs in haemolymph samples collected from larvae infected with *E. coli* was reflected by their antimicrobial activities in inhibition-zone assays (nos 5 to 8; Fig. 2B). Comparable reactions were observed upon challenge of drone larvae with other elicitors such as LPS from *E. coli*, peptidoglycan from *Micrococcus luteus* and laminarin from *Laminaria digitata* (not shown). Aseptic wounding (PBS) of 6-day-old larvae (but not of 7- or 8-day-old larvae) resulted in the induction of antimicrobial compounds as deduced from the generation of clear inhibition zones by the corresponding haemolymph samples (nos 3 and 4; Fig. 2B), however, the two specific AMPs hymenoptaecin and defensin 1 were hardly visible in polyacrylamide gels (nos 3 and 4; Fig. 2A). It should be noted in this context that no intrinsic antimicrobial activity could be detected in untreated drone larvae (nos 1 and 2; Fig. 2)

Developmental-specific expression of haemolymph proteins in adult drones

Newly emerged drones were marked individually and reintroduced into the original colony. Haemolymph samples were collected from young (1–12 days old) and old drones (1–6 weeks old) for subsequent proteomic analyses. In 1- to 2-day-old adult drones (Fig. 3A, supplementary material Table S1), the major haemolymph proteins are the precursor apolipophorin-I (ApoLp-I) and its processing product ApoLp-II (the latter lacking the N-terminal amino acids 1 to 747) that mainly act as lipid transporters in the body fluid (Weers and Ryan, 2006). Additionally, transferrin is a prominent haemolymph protein. It belongs to a family of iron-binding proteins that – in contrast to ferritins – are entailed in iron transport. Moreover, transferrin mediates a number of vital reactions and appears to also play a role in defence reactions (Kucharski and Maleszka, 2003; Dunkov and Georgieva, 2006).

Vitellogenin, a glycoprotein of approximately 200 kDa, is present in the haemolymph of adult drones at very low concentrations and it is produced only between 3 and 14 days after emergence (Fig. 3A,C). This expression profile differs considerably from that of adult worker bees as discussed below. Two haemolymph proteins, i.e. α-glucosidase II and a protein with a molecular mass of

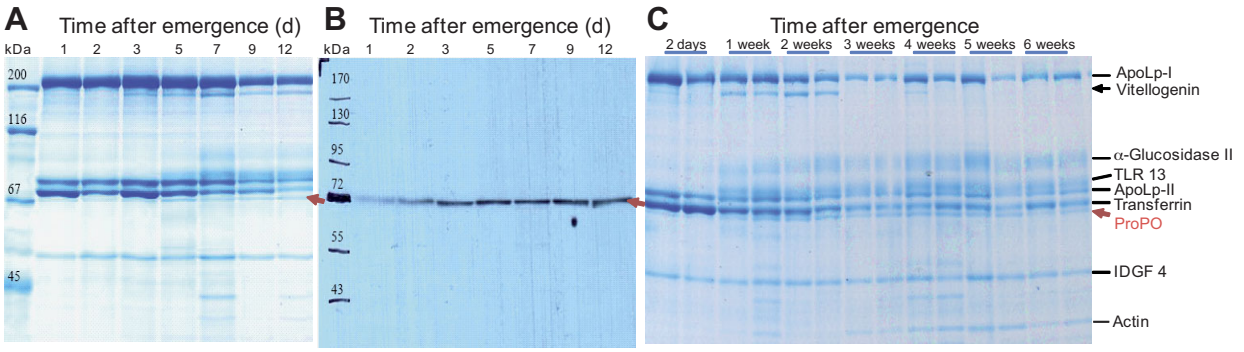

Fig. 3. Age-dependent expression of major haemolymph proteins of adult honey bee drones. (A,C) Gel electrophoretic analysis of haemolymph proteins. Newly emerged drones were collected from a drone comb, labelled individually with a paint marker and placed back into the original colony. At the indicated times, three to five marked drones were removed from the hive for subsequent haemolymph extraction. Aliquots (1 μl) were mixed with dissociation buffer and applied onto a 10% polyacrylamide/0.1% SDS gel. (B) For the immunological identification of prophenoloxidase (proPO), proteins of gel A were blotted on a PVDF membrane and probed with an antibody directed against a proPO peptide. ApoLp-I (gil328780884) and ApoLp-II, apolipophorin (gil66513966); vitellogenin (gil58585104); α-glucosidase II (gil94400901); TLR 13, toll-like receptor 13 (gil110755367); transferrin (gil58585086); ProPO, prophenoloxidase (gil58585196; indicated by a red arrow); IDGF 4, imaginal disc growth factor 4 (gil66514614); actin, actin related protein 1 (gil297591987).

approximately 75 kDa assigned as toll-like receptor 13-like isoform 1 (TLR 13), are first detected in 5- to 7-day-old drones and are continuously expressed throughout the entire lifespan of adult drones (Fig. 3A,C). α-Glucosidases catalyse the liberation of an α-glucosyl residue from a broad spectrum of substrates such as sucrose and maltose. Three kinds of glucosidases were purified from honey bees. It was immunologically confirmed that α-glucosidase I is present in the ventriculus, α-glucosidase II is present in the ventriculus and the haemolymph, and α-glucosidase III is present in the hypopharyngeal gland, the latter enzyme being identical with the α-glucosidase present in honey (Kubota et al., 2004). The physiological function of α-glucosidase II is not yet known. However, it should be noted that the first appearance of α-glucosidase II in the haemolymph of adult drones coincides with first flight activities (Fukuda and Ohtani, 1977), which require large amounts of glucose (Gmeinbauer and Crailsheim, 1993). Similarly, the enhanced occurrence of TLR 13 in the haemolymph of adult drones is also coupled to late developmental stages (Fig. 3). In this context it should be noted that TLR 13 is a major haemolymph protein of forager bees (K. Azzami, unpublished observation). It is not known which function(s) TLR 13 exerts in insects. It is a novel and poorly characterized member of the Toll-like receptor family whose expression in mammalian cell lines has not revealed a direct involvement in the innate immune response to infections (Shi et al., 2009).

Phenoloxidase is a vital enzyme essential for a number of processes such as cuticular hardening (sclerotization), pigmentation, wound healing and nodulation. It is present as an inactive precursor, called proPO zymogen, and is activated by a cascade of serine proteases (Cerenius et al., 2008). ProPO is first detected in the haemolymph of newly emerged drones (1–2 days old) and is then present at an almost constant level until the end of the lifespan of adult drones, as verified by western blotting (Fig. 3B) and MS/MS analyses of excised protein samples.

Adult young drones respond to septic injury with a broad array of immune-responsive proteins

In the haemolymph of young drones, the same two AMPs, hymenoptaecin and defensin 1, already identified in drone larvae upon challenge with viable *E. coli* cells (Fig. 2) were induced to a high level 24 and 48 h post-infection (p.i.) (Fig. 4A, supplementary material Table S2). In accordance with these results, inhibition-zone assays disclosed the presence of active antimicrobial compounds (Fig. 4B). In contrast to drone larvae, a number of immune-

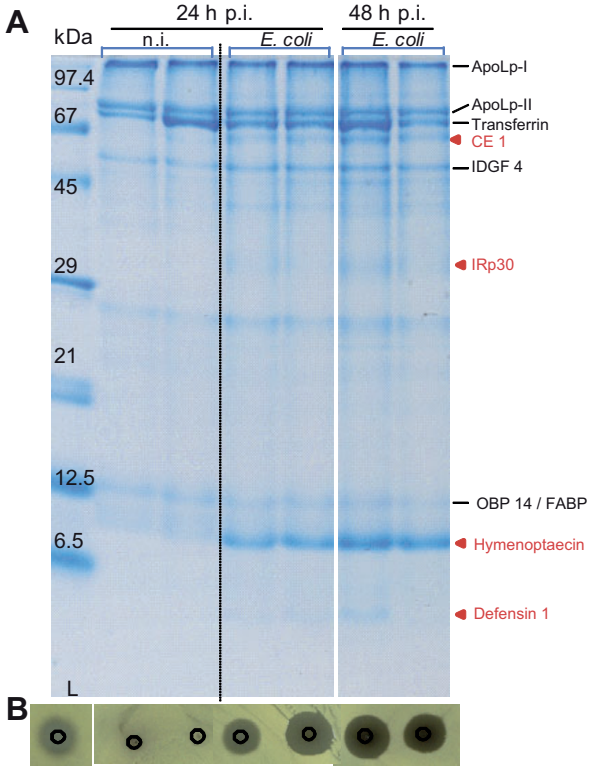

Fig. 4. Characterization of immune-responsive proteins in young adult honey bee drones by one-dimensional gel electrophoresis. (A) Newly emerged drones were left untreated (n.i.) or challenged with 10^5 viable *E. coli* cells. Haemolymph samples were collected 24 and 48 h p.i. followed by proteome analysis of two replicates. An aliquot of these samples (1 μl) was mixed with dissociation buffer and applied onto a 15% polyacrylamide/0.1% SDS gel. Induced AMPs and immune-responsive proteins are indicated by red arrowheads. (B) Aliquots of the same haemolymph samples were applied on agar plates together with *M. flavus* as indicator bacteria. CE 1, carboxylesterase (gil66512983); IRp30 (gil66507096); hymenoptaecin (gil58585174); defensin 1 (gil37703274).

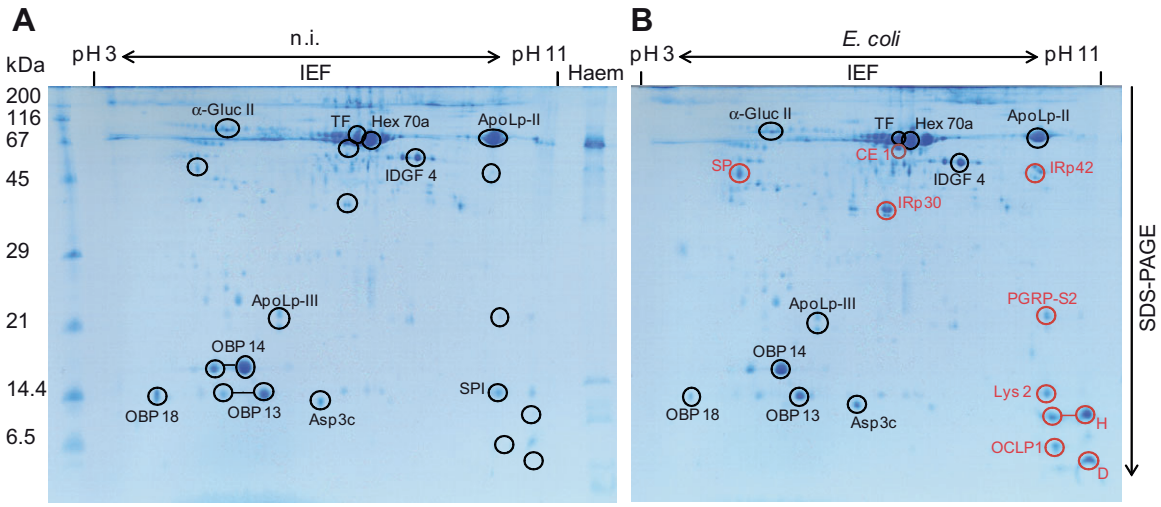

Fig. 5. Characterization of immune-responsive proteins in young adult honey bee drones by two-dimensional gel electrophoresis. Six-day-old drone adults were left untreated (A) or challenged with 10^5 viable *E. coli* cells (B). Haemolymph samples were collected 24 h p.i. and soluble haemolymph proteins were analysed by two-dimensional gel electrophoresis. Proteins were separated by isoelectric focusing (IEF) in the first dimension and by SDS-PAGE in the second dimension. Encircled gel spots were excised and the proteins were identified by subsequent MS/MS analyses. Differentially expressed immune-responsive proteins are marked by red circles in B and empty rings in A. ApoLp-II, apolipophorin (gil66513966); ApoLp-III, apolipophorin (gil166795901); α-Gluc II, α-glucosidase II (gil94400901); TF, transferrin (gil58585086); Hex 70a, hexamerin 70a (gil148357839); CE 1, carboxylesterase (gil66512983); IDGF 4, imaginal disc growth factor 4 (gil66514614); SP, serine protease (gil110758534); IRp42 (gil66519003); IRp30 (gil66507096); PGRP-S2, peptidoglycan recognition protein-S2 (gil254910931); OBP13, odorant binding protein 13 (gil94158810); OBP 14, odorant binding protein 14 (gil94158822); OBP 18, odorant binding protein 18 (gil94158830); Asp3c, antennal-specific protein 3c (gil58585106); Lys 2, lysozyme 2 (gil11762162); SPI, serine protease inhibitor (similar to gil340725718); OCLP1, ω-conotoxin-like protein 1 (gil110756431); H, hymenoptaecin (gil58585174); D, defensin 1 (gil37703274). Haem, haemolymph control sample without IEF separation.

responsive proteins were additionally induced or upregulated, e.g. carboxylesterase (CE 1) and a protein with an unknown function (IRp30). For a better resolution, we separated haemolymph proteins by two-dimensional gel electrophoresis. This sensitive technique revealed some novel immune-responsive proteins, among them a trypsin-like serine protease (SP), PGRP-S2, another protein of unknown function (IRp42) with an approximate molecular mass of 42 kDa, and lysozyme 2 (Fig. 5B, supplementary material Table S2). A protein migrating to a similar position as lysozyme 2 in two-dimensional polyacrylamide gels was detected in haemolymph samples collected from non-infected adult drones. It was identified as a putative serine protease inhibitor of the kazal type (SPI) by MS/MS analyses, suggesting that this type of protease inhibitor is downregulated, whereas lysozyme 2 is upregulated upon bacterial challenge.

A small peptide whose expression is readily induced in immunized adult drones is assigned as a hypothetical protein with an approximate molecular mass of 8.2 kDa in the NCBI database. In fact, this specific peptide has been recently shown to belong to a family of toxin-like proteins that exhibit similarity to ion channel inhibitors. It was tentatively called ω-conotoxin-like protein 1 (OCLP1). Interestingly, it was found to be transcriptionally expressed predominantly in the honey bee brain (Kaplan et al., 2007). In a comprehensive study of differential gene expression of the honey bees *Apis mellifera* and *Apis cerana* induced by *Varroa destructor* infection, it was shown by dot blot analyses that transcription of the OCLP1 gene (indicated as hypothetical protein LOC725074) was downregulated in *A. mellifera* prepupae (Zhang et al., 2010).

Furthermore, several haemolymph proteins were resolved by two-dimensional gel electrophoresis that were not correlated with microbial challenge, among them apolipophorin-III (ApoLp-III), another component of the lipid transport system (Weers and Ryan, 2006), antennal-specific protein 3c (ASP3c), a soluble chemosensory protein (Briand et al., 2002; Forêt et al., 2007), and three members of the odorant binding-like protein (OBP) family, OBP 13, OBP 14 and OBP 18. Of the three OBPs identified in the haemolymph of adult drones, OBP 14 was present also in drone larvae (Fig. 2) whereas OBP 13 and OBP18 were not detected in larval haemolymph (data not shown). The honey bee genome encodes 21 OBP genes, only nine of which are specifically upregulated in antennae. The remaining genes are expressed either ubiquitously or in specialized tissues during development, supporting the notion that OBPs are not restricted to olfaction (Forêt and Maleszka, 2006). Similar observations were made by Calvello et al. (Calvello et al., 2005), who found that OBPs are expressed in different organs in relation to age, caste and rank in other social hymenopterans such as *Polistes dominulus*.

Old drones maintain the ability for humoral immune reactions

As stated above, young drones (1–6 days old) responded with the upregulation of a number of proteins and/or peptides upon bacterial challenge (Figs 4, 5). We then asked the question whether old adult drones (1–6 weeks old) react similarly to septic injury. Hence, marked drones were collected from the hive and infected artificially with viable *E. coli* cells. Drones that were left untreated (n.i.) served as controls. Proteomic analyses of selected haemolymph samples revealed that the level of induced AMPs, i.e. hymenoptaecin and defensin 1, as well as the amount of some immune-responsive proteins such as carboxylesterase (CE 1) and IRp30, decreased in long-lived drones upon bacterial challenge (Fig. 6B). However, the ability to react with the synthesis of antimicrobial compounds upon septic injury is maintained throughout the entire lifespan of adult drones as manifested by the inhibition-zone assay. Large inhibition zones were produced by haemolymph samples derived from old drones (1–6 weeks old)

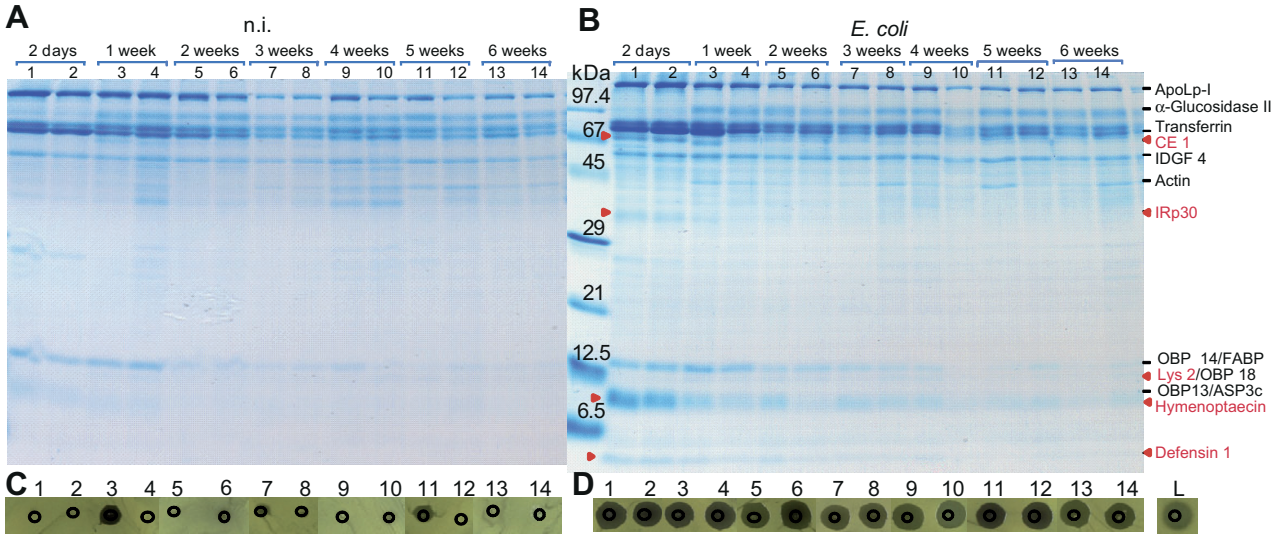

Fig. 6. Age-dependent expression of humoral immune responses of adult honey bee drones. (A,B) Newly emerged drones were collected from a drone comb, labelled with a paint marker and placed back into the original colony. At the indicated time, 10 marked drones were removed from the hive and divided into two groups: one group was left untreated (n.i.), and the other was challenged with 10^5 viable *E. coli* cells and labelled additionally with a paint marker. Untreated and treated drones were kept together with an equal number of worker bees in a small cage at 30°C, supplemented with 50% (v/v) ApiInvert *ad libitum*. Haemolymph samples were collected 24 h p.i. An aliquot (1 μl) of haemolymph samples was mixed with dissociation buffer and applied onto a 15% polyacrylamide/0.1% SDS gel. Induced AMPs and immune-responsive proteins are indicated by red arrowheads. (C,D) Aliquots of the same haemolymph samples were applied onto agar plates together with *M. flavus* as indicator bacteria.

challenged with *E. coli*, whereas no antimicrobial activity was detected in haemolymph samples originating from non-treated old drones (Fig. 6C,D). Similar results were obtained if *E. coli* instead of *M. flavus* were used as indicator bacteria in the inhibition-zone assay (data not shown).

Nodulation reactions are differentially induced in drone larvae and adults

Nodulation is a predominant cellular immune reaction in many insects, and is activated by bacteria and fungi and involves the interaction between circulating haemocytes and microorganisms (Horohov and Dunn, 1983). It was recently demonstrated by Bedick et al. (Bedick et al., 2001) that this septic cellular defence reaction is also expressed in newly emerged worker bees artificially infected with freeze-dried bacteria (*Serratia marcescens*). We injected viable *E. coli* cells into the haemocoel of young adult drones and assessed the formation of nodules at selected times post-injection. The first melanised nodules were visible 2 h p.i., reached a maximum of approximately 1000 nodules per drone between 10 and 20 h p.i. and thereafter the total number of visible nodules remained constant up to 72 h p.i. A typical image of nodule formation 24 h post-infection of 10^5 *E. coli* cells is shown in Fig. 7B. The abdominal tergites of infected drones were removed so that the haemocoel is exposed. Most melanised nodules are attached along the dorsal vessel.

We also examined nodule reactions in drone larvae upon bacterial challenge. Septic injury of 7- to 8-day-old larvae revealed no nodule formation at all. A weak response was observed in 9-day-old drone larvae that were challenged with 10^5 *E. coli* cells. An average of 35 nodules per larva was detected in a total of 18 infected individuals 24 h p.i. (Fig. 7D).

DISCUSSION

Drones may encounter microbial infections inside and outside the hive (Bailey and Ball, 1991). Yet little attention has been paid so far to their immunocompetence, although their survival is vital for the maintenance of the bee colony. To address this question, we studied the defence reactions of drones at different life stages upon artificial bacterial infection. As parameters for immune strength, we investigated: (1) the production of AMPs as an indication for an active humoral immune system, (2) the formation of nodules as a representative cellular immune response and (3) the expression of proPO zymogen. To our surprise, we found that male drones utilize the same repertory of defence reactions as female workers at each examined developmental stage.

A humoral immune response to bacterial challenge was activated throughout all life stages from drone larvae to adult drones. The induction of the humoral immune system was confirmed by either inhibition-zone assays or proteomic analyses of haemolymph proteins (Figs 2, 4–6). The inhibition-zone assay revealed the total antimicrobial activity present in collected haemolymph samples, whereas gel electrophoresis allowed the identification of specific AMPs. Hymenoptaecin is by far the most prominent AMP detectable in polyacrylamide gels. The high titres induced upon microbial infection correlate with a high and persistent transcriptional activity of the corresponding gene (Casteels-Josson et al., 1994). Hymenoptaecin plays a central role among the known honey-bee-specific AMPs because it affects a wide variety of Gram-positive and Gram-negative bacteria (Casteels et al., 1993). Expression of hymenoptaecin is mediated by the Imd signalling cascade, as recently revealed by an RNA interference study (Schlüns and Crozier, 2007). Hence, its production is a clear indication of the activation of at least one signalling pathway of the humoral immune system.

Adult drones responded with a number of additional reactions to bacterial challenge that were not detected in larvae. Several polypeptides were identified (Fig. 5), particularly by two-dimensional gel electrophoresis, whose synthesis was upregulated after injection of viable *E. coli* bacteria into the haemocoel of young drones. Among these immune-responsive proteins (IRps) are two of unknown function (i.e. IRp42 and IRp30) that are assigned as

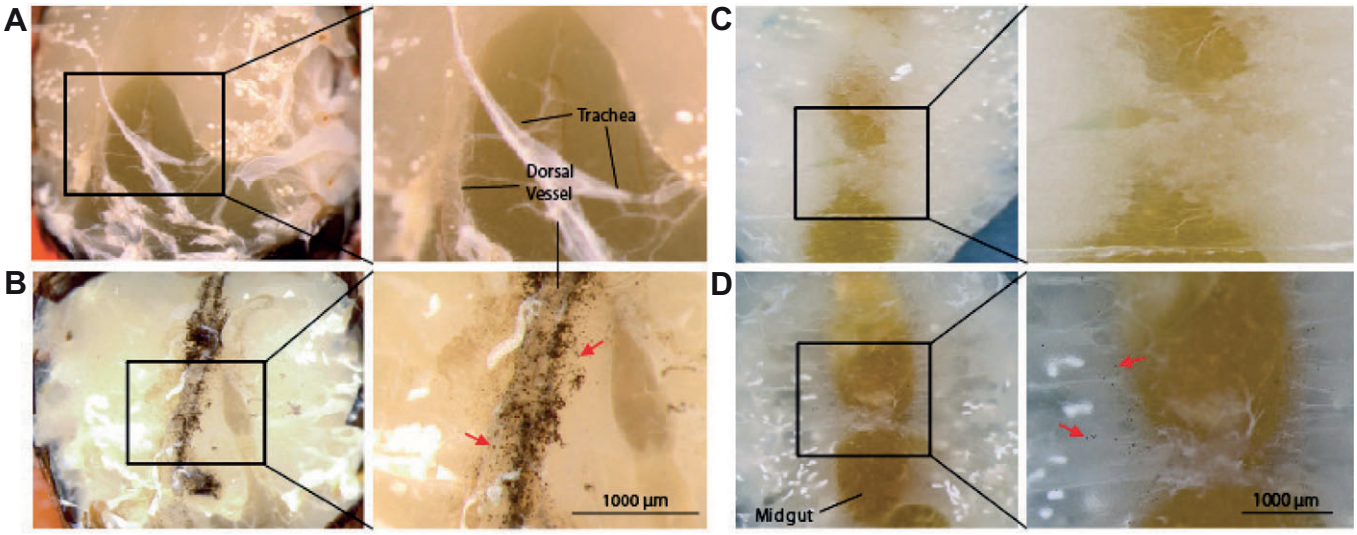

Fig. 7. Comparison of cellular immune responses of honey bee drone larvae and adult drones. Newly emerged adult drones (A,B) and 9-day-old drone larvae (C,D) were either left untreated (A,C) or challenged with 10^5 viable *E. coli* cells (B,D). Groups of 10 adult drones together with an equal number of workers were kept in small cages at 30°C. A total of 10 untreated and 30 infected drone larvae were transferred to tissue culture plates and kept in an incubator at 35°C. Upon 24 h post-injection, the abdominal tergites of adult drones were removed (A,B) and the body cavity of drone larvae was exposed (C,D) followed by inspection for the presence of melanised nodules (indicated by red arrows). Photomicrographs were made with an Olympus SZX7 stereomicroscope equipped with an Olympus UC30 camera. Magnification was ×20 for the complete abdomen and ×32 for the detailed view.

hypothetical proteins in the NCBI database. However, detailed studies of the expression of IRp30 in adult worker bees have revealed that it is simultaneously upregulated with a specific member of the carboxylesterase family (see below) upon treatment with *E. coli* and other elicitors. Interestingly, IRp30 belongs to a protein family distributed exclusively among social Hymenoptera (Albert et al., 2011). For the remaining IRps, putative annotations are available. The upregulation of PGRP-S2 upon septic injury has already been described in young adult worker bees (Evans et al., 2006; Randolt et al., 2008). PGRPs are major components of pathogen recognition. They either function as recognition proteins for pathogen-associated molecular patterns or may exert amidase activity and then act as scavengers that degrade bacterial cell wall components (Steiner, 2004). Another polypeptide whose synthesis was increased in young adult drones (Figs 4, 5) as well as in newly emerged worker bees upon bacterial challenge (Randolt et al., 2008) is a member of the carboxylesterase family (CE 1). Carboxylesterases belong to a large family of type B esterases and lipases with multiple functions (Trenczek, 1998). The third peptide associated with an antibacterial response in adult drones is a trypsin-like SP. It has been suggested that this protease participates in the proteolytic cascade that activates the inactive proPO zymogen (Chan et al., 2006; Zou et al., 2006). Another newly identified immune-responsive protein (OCLP1) has been assigned as a toxin-like peptide by Kaplan et al. (Kaplan et al., 2007). Our discovery of this small peptide in the haemolymph of adult drones together with its induction after bacterial infection suggests that this toxin-like peptide exhibits antibacterial activity in addition to cytotoxic features.

A total of three lysozyme genes have been annotated in the honey bee genome (Kunieda et al., 2006; Evans et al., 2006), two of which are expressing lysozymes of the chicken (c)-type (lys 1 and lys 2), whereas the third is coding for an invertebrate (i)-specific lysozyme (lys i or lys 3). We detected lys 2 in haemolymph samples collected from immunized adult drones (Fig. 5B), indicating that this lysozyme type is induced upon septic injury. All of the lysozymes are characterized by their ability to hydrolyse bacterial peptidoglycan, thereby playing an important role in the antibacterial defence (Bachali et al., 2002). The bacterial affinities of the various lysozyme types may be very different. For example, a comparative study on lepidopteran lysozymes has revealed that they have strong activities against Gram-positive bacteria and in addition express – in contrast to the chicken lysozyme – weak activities against Gram-negative bacteria (Yu et al., 2002).

Nodulation is a cellular defence reaction to bacterial infections in insects (Horohov and Dunn, 1983) that is mediated by eicosanoids (Bedick et al., 2001). In honey bees, nodule formation apparently is limited to a narrow range of the lifespan. Drone larvae responded with a very weak nodulation reaction compared with young adults (Fig. 7) and we also observed a significant reduced ability of nodule formation upon bacterial challenge in 11-day-old drones (H.G., unpublished). Similar reactions have been elucidated in worker bees. Bacterial infection of newly emerged worker bees resulted in a pronounced nodulation response whereas honey bee foragers did not evoke nodulation reactions (Bedick et al., 2001). The question then arises, how do old drones and workers cope with microbial infections in the absence of one major component of the cellular response? A number of reports have recorded a strong reduction in the haemocyte number of old honey bees of all castes (Fluri et al., 1977; Amdam et al., 2004; Schmid et al., 2008; Wilson-Rich et al., 2008), supporting the assumption of a reduced cellular immunocompetence in old honey bees. However, the possibility remains that the decline in the total number of haemocytes might represent only the reduction of a subgroup of haemocytes and thus does not affect all cellular immune reactions. In fact, Wilson-Rich et al. (Wilson-Rich et al., 2008) observed no differences in the encapsulation response across all developmental stages from larvae to adult worker honey bees.

Phenoloxidase-catalysed melanisation of pathogens is a key element of invertebrate defences (Cerenius et al., 2008) and may compensate for the loss of specific cellular defence mechanisms.

In larvae and pupae of worker bees, the amount of proPO transcripts is low (Lourenço et al., 2005). The level of proPO transcripts as well as phenoloxidase activity were found to increase steadily with age in adult workers and queens, finally reaching a plateau (Wilson-Rich et al., 2008; Schmid et al., 2008; Laughton et al., 2011). In contrast, PO activity levels remained rather constant in adult drones (Schmid et al., 2008), which is consistent with our finding of a rather stable concentration of proPO zymogen in adult drones (Fig. 3).

The differential expression of major haemolymph proteins in drone larvae and adult drones (Figs 1, 3) resembles the observed developmental-specific protein expression in the haemolymph of worker bees (Chan et al., 2006; Chan and Foster, 2008; Randolt et al., 2008), with one exception that concerns the vitellogenin production. Vitellogenin is present in the haemolymph of adult drones only for a limited period of the lifespan, between 3 and 14 days after emergence, and at quite low concentrations (Fig. 3) (Trenczek et al., 1989), whereas the total amount of vitellogenin in the haemolymph of young worker bees is much higher and elevated levels of vitellogenin are maintained in old hive bees and winter bees (Amdam et al., 2005a; Azzami et al., 2009). It has been implicated that longevity and immunocompetence of honey bee queens and workers, respectively, are linked to high levels of vitellogenin (Amdam et al., 2004; Amdam et al., 2005b; Corona et al., 2007). Yet, drone larvae and adult drones are capable of exerting a variety of immune reactions such as nodulation and the induction of AMPs in the complete absence of vitellogenin (Figs 2, 4, 6, 7), suggesting that most, if not all, components of the innate immune system do not rely on the expression of vitellogenin.

Sex- and caste-specific differences in immunocompetence have been observed in some insects and have sometimes led to contradictory statements depending on the parameters chosen for the study of immune reactions (Moret and Schmid-Hempel, 2001; Baer et al., 2005; Baer and Schmid-Hempel, 2006; Laughton et al., 2011). Our results undoubtedly imply that honey bee drones are capable of exerting diverse defence reactions upon bacterial infection, which should ensure their survival under high pathogen pressure.

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REFERENCES

Albert, Š., Gätschenberger, H., Azzami, K., Gimple, O., Grimmer, G., Sumner, S., Fujiyuki, T., Tautz, J. and Mueller, M. J. (2011). Evidence of a novel immune responsive protein in the Hymenoptera. *Insect Biochem. Mol. Biol.* **41**, 968-981.

Amdam, G. V., Simões, Z. L. P., Hagen, A., Norberg, K., Schrøder, K., Mikkelsen, Ø., Kirkwood, T. B. L. and Omholt, S. W. (2004). Hormonal control of yolk precursor vitellogenin regulates immune function and longevity in honeybees. *Exp. Gerontol.* **39**, 767-773.

Amdam, G. V., Norberg, K., Omholt, S. W., Kryger, P., Lourenço, A. P., Bitondi, M. M. G. and Simões, Z. L. P. (2005a). Higher vitellogenin concentrations in honey bee workers may be an adaption to life in temperate climates. *Insect. Soc.* **52**, 316-319.

Amdam, G. V., Aase, A. L. T. O., Seehuus, S.-C., Fondrk, M. K., Norberg, K. and Hartfelder, K. (2005b). Social reversal of immunosenescence in honey bee workers. *Exp. Gerontol.* **40**, 939-947.

Azzami, K., Gätschenberger, H., Gimple, O., Beier, H. and Tautz, J. (2009). Do winter bees have an immune system that is weaker than that of summer bees? *Apidologie* **40**, 660.

Bachali, S., Jager, M., Hassanin, A., Schoentgen, F., Jollès, P., Fiala-Medioni, A. and Deutsch, J. S. (2002). Phylogenetic analysis of invertebrate lysozymes and the evolution of lysozyme function. *J. Mol. Evol.* **54**, 652-664.

Baer, B. and Schmid-Hempel, P. (2006). Phenotypic variation in male and worker encapsulation response in the bumblebee *Bombus terrestris*. *Ecol. Entomol.* **31**, 591-596.

Baer, B., Krug, A., Boomsma, J. J. and Hughes, W. O. H. (2005). Examination of the immune responses of males and workers of the leaf-cutting ant *Acromyrmex echinatior* and the effect of infection. *Insect. Soc.* **52**, 298-303.

Bailey, L. and Ball, B. V. (1991). *Honey Bee Pathology*. London: Academic Press.

Bedick, J. C., Tunaz, H., Nor Aliza, A. R., Putman, S. M., Ellis, M. D. and Stanley, D. W. (2001). Eicosanoids act in nodulation reactions to bacterial infections in newly emerged adult honey bees, *Apis mellifera*, but not in older foragers. *Comp. Biochem. Physiol.* **130C**, 107-117.

Behrens, D., Forsgren, E., Fries, I. and Moritz, R. F. A. (2007). Infection of drone larvae (*Apis mellifera*) with American foulbrood. *Apidologie* **38**, 281-288.

Briand, L., Swasdipan, N., Nespoulous. C., Bézirard, V., Blon, F., Huet, J.-C., Ebert, P. and Pernollet, J.-C. (2002). Characterization of a chemosensory protein (ASP3c) from honeybee (*Apis mellifera* L.) as a brood pheromone carrier. *Eur. J. Biochem.* **269**, 4586-4596.

Burmester, T. and Scheller, K. (1999). Ligands and receptors: common theme in insect storage protein transport. *Naturwissenschaften* **86**, 468-474.

Calvello, M., Brandazza, A., Navarrini, A., Dani, F. R., Turilazzi, S., Felicioli, A. and Pelosi. P. (2005). Expression of odorant-binding proteins and chemosensory proteins in some Hymenoptera. *Insect Biochem. Mol. Biol.* **35**, 297-307.

Casteels, P., Ampe, C., Jacobs, F., Vaeck, M. and Tempst, P. (1989). Apidaecins: antibacterial peptides from honeybees. *EMBO J.* **8**, 2387-2391.

Casteels, P., Ampe, C., Riviere, L., Van Damme, J., Elicone, C., Fleming, M., Jacobs, F. and Tempst, P. (1990). Isolation and characterization of abaecin, major antibacterial response peptide in the honeybee (*Apis mellifera*). *Eur. J. Biochem.* **187**, 381-386.

Casteels, P., Ampe, C., Jacobs, F. and Tempst, P. (1993). Functional and chemical characterization of hymenoptaecin, an antibacterial polypeptide that is infection-inducible in the honeybee (*Apis mellifera*). *J. Biol. Chem.* **268**, 7044-7054.

Casteels-Josson, K., Zhang, W., Capaci, T., Casteels, P. and Tempst, P. (1994). Acute transcriptional response of the honeybee peptide-antibiotics gene repertoire and required post-translational conversion of the precursor structures. *J. Biol. Chem.* **269**, 28569-28575.

Cerenius, L., Lee, B. L. and Söderhäll, K. (2008).The proPO-system: pros and cons for its role in invertebrate immunity. *Trends Immunol.* **29**, 263-271.

Chan, Q. W. T. and Foster, L. J. (2008). Changes in protein expression during honey bee larval development. *Genome Biol.* **9**, R156.

Chan, Q. W. T., Howes, C. G. and Foster, L. J. (2006). Quantitative conmparison of caste differences in honeybee hemolymph. *Mol. Cell. Proteomics* **5**, 2252-2262.

Corona, M., Velarde, R. A., Remolina, S., Moran-Lauter, A., Wang. Y., Hughes, K. A. and Robinson, G. E. (2007). Vitellogenin, juvenile hormone, insulin signalling, and queen honey bee longevity. *Proc. Natl. Acad. Sci. USA* **104**, 7128-7133.

Danty, E., Arnold, G., Burmester, T., Huet, J.-C., Huet, D., Pernollet, J. C. and Masson, C. (1998). Identification and developmental profiles of hexamerins in antenna and hemolymph of the honeybee, *Apis mellifera*. *Insect Biochem. Mol. Biol.* **28**, 387-397.

Dunkov, B. and Georgieva, T. (2006). Insect iron binding proteins: Insights from the genomes. *Insect Biochem. Mol. Biol.* **36**, 300-309.

Evans, J. D., Aronstein, K., Chen, Y. P., Hetru, C., Imler, J.-L., Jiang, H., Kanost, M., Thompson, G. J., Zou, Z. and Hultmark, D. (2006). Immune pathways and defence mechanisms in honey bees *Apis mellifera*. *Insect Mol. Biol.* **15**, 645-656.

Fluri, P., Wille, H., Gerig, L. and Lüscher, M. (1977). Juvenile hormone, vitellogenin and haemocyte composition in winter worker honeybees (*Apis mellifera*). *Experientia* **33**, 1240-1241.

Forêt, S. and Maleszka, R. (2006). Function and evolution of a gene family encoding odorant binding-like proteins in a social insect, the honey bee (*Apis mellifera*). *Genome Res.* **16**, 1404-1413.

Forêt, S., Wanner, K. W. and Maleszka, R. (2007). Chemosensory proteins in the honey bee: insights from the annotated genome, comparative analyses and expressional profiling. *Insect Biochem. Mol. Biol.* **37**, 19-28.

Fukuda, H. and Ohtani, T. (1977). Survival and life-span of drone honeybees. *Res. Popul. Ecol.* **19**, 51-68.

Gmeinbauer, R. and Crailsheim, K. (1993). Glucose utilization during flight of honeybee (*Apis mellifera*) workers, drones and queens. *J. Insect Physiol.* **39**, 959-967.

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

Horohov, D. W. and Dunn, P. E. (1983). Phagocytosis and nodule formation by hemocytes of M*anduca sexta* larvae following injection of *Pseudomonas aeruginosa*. *J. Invertebr. Pathol.* **41**, 203-213.

Hrassnigg, N. and Crailsheim, K. (2005). Differences in drone and worker physiology in honeybees (*Apis mellifera*). *Apidologie* **36**, 255-277.

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

Kaplan, N., Morpurgo, A. and Linial, M. (2007). Novel families of toxin-like peptides in insects and mammals: a computational approach. *J. Mol. Biol.* **369**, 553-566.

Kawamura, K., Shibata, T., Saget, O., Peel, D. and Bryant, P. J. (1999). A new family of growth factors produced by the fat body and active on *Drosophila* imaginal disc cells. *Development* **126**, 211-219.

Klaudiny, J., Albert, S., Bachanova, K., Kopernicky, J. and Simuth, J. (2005). Two structurally different defensin genes, one of them encoding a novel defensin isoform, are expressed in honeybee *Apis mellifera*. *Insect Biochem. Mol. Biol.* **35**, 11-22.

Kubota, M., Tsuji, M., Nishimoto, M., Wongchawalit, J., Okuyama, M., Mori, H., Matsui, H., Surarit, R., Svasti, J., Kimura, A. et al. (2004). Localization of α-

glucosidases I, II, III in organs of European honeybees, *Apis mellifera* L., and the origin of α-glucosidase in honey. *Biosci. Biotechnol. Biochem.* **68**, 2346-2352.
Kucharski, R. and Maleszka, R. (2003). Transcritional profiling reveals multifunctional roles for transferrin in the honeybee, *Apis mellifera*. *J. Insect Sci.* **3**, 27.
Kunieda, T., Fujiyuki, T., Kucharski, R., Foret, S., Ament, S. A., Toth, A. L., Ohashi, K., Takeuchi, H., Kamikouchi, A., Kage, E. et al. (2006). Carbohydrate metabolism genes and pathways in insects: insights from the honey bee genome. *Insect Mol. Biol.* **15**, 563-576.
Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.
Laughton, A. M., Boots, M. and Siva-Jothy, M. T. (2011). The ontogeny of immunity in the honey bee, *Apis mellifera* L. following an immune challenge. *J. Insect Physiol.* **57**, 1023-1032.
Lemaitre, B. and Hofmann, J. (2007). The host defense of *Drosophila melanogaster. Annu. Rev. Immunol.* **25**, 697-743.
Lourenço, A. P., Zufelato, M. S., Bitondi, M. M. G. and Simões, Z. L. P. (2005). Molecular characterization of a cDNA encoding prophenoloxidase and its expression in *Apis mellifera. Insect Biochem. Mol. Biol.* **35**, 541-552.
Martins, J. R., Nunes, F. M. F., Cristino, A. S., Simoes, Z. L. P. and Bitondi, M. M. G. (2010). The four hexamerin genes in the honey bee: structure, molecular evolution and function deduced from expression patterns in queens, workers and drones. *BMC Mol. Biol.* **11**, 23.
Moret, Y. and Schmid-Hempel, P. (2001). Immune defence in bumble-bee offspring. *Nature* **414**, 506.
Peng, Y.-S. C., Mussen, E., Fong, A., Montague, M. A. and Tyler, T. (1992). Effects of chlortetracycline of honey bee worker larvae reared *in vitro*. *J. Invertebr. Path.* **60**, 127-133.
Randolt, K., Gimple, O., Geissendörfer, J., Reinders, J., Prusko, C., Mueller, M. J., Albert, S., Tautz, J. and Beier, H. (2008). Immune-related proteins induced in the hemolymph after aseptic and septic injury differ in honey bee worker larvae and adults. *Arch. Insect. Biochem. Physiol.* **69**, 155-167.
Schlüns, H. and Crozier, R. H. (2007). Relish regulates expression of antimicrobial peptide genes in the honeybee, *Apis mellifera*, shown by RNA interference. *Insect Mol. Biol.* **16**, 753-759.
Schmid, M. R., Brockmann, A., Pirk, C. W. W., Stanley, D. W. and Tautz, J. (2008). Adult honeybees (*Apis mellifera* L.) abandon hemocytic, but not phenoloxidase-based immunity. *J. Insect Physiol.* **54**, 439-444.
Shi, Z., Cai, Z., Wen, S., Chen, C., Gendro, C., Sanchez, A., Patterson, K., Fu, S., Yang., Wildman, D. et al. (2009). Transcriptional regulation of the novel Toll-like receptor Tlr13. *J. Biol. Chem.* **284**, 20540-20547.
Shipman, B. A., Ryan, R. O., Schmidt, J. O. and Law, J. H. (1987). Purification and properties of a very high density lipoprotein from the haemolymph of the honeybee *Apis mellifera*. *Biochemistry* **26**, 1885-1889.
Steiner, H. (2004). Peptidoglycan recognition proteins: on and off switches for innate immunity. *Immunol. Rev.* **198**, 83-96.
Trenczek, T. (1998). Endogenous defense mechanisms of insects. *Zoology* **101**, 298-315.
Trenczek, T., Zillikens, A. and Engels, W. (1989). Developmental patterns of vitellogenin haemolymph titre and rate of synthesis in adult drone honey bees (*Apis mellifera*). *J. Insect Physiol.* **35**, 475-481.
Weers, P. M. M. and Ryan, R. O. (2006). Apolipophorin III: Role model apolipoprotein. *Insect Biochem. Mol. Biol.* **36**, 231-240.
Wilson-Rich, N., Dres, S. T. and Starks, P. T. (2008). The ontogeny of immunity: Development of innate immune strength in the honey bee (*Apis mellifera*). *J. Insect Physiol.* **54**, 1392-1399.
Winston, M. L. (1987). *The Biology of the Honeybee*. Cambridge, MA: Harvard University Press.
Yu, K. H., Kim, K. N., Lee, J. H., Lee, H. S., Kim, S. H., Cho, K. Y., Nam, M. H. and Lee, I. H. (2002). Comparative study on characteristics of lysozymes from the hemolymph of three lepidopteran larvae, *Galleria mellonella, Bombyx mori, Agrius convolvuli*. *Dev. Comp. Immunol.* **26**, 707-713.
Zhang, Y., Liu, X., Zhang, W. and Han, R. (2010). Differential gene expression of the honey bees *Apis mellifera* and *A. cerana* induced by *Varroa destructor* infection. *J. Insect Physiol.* **56**, 1207-1218.
Zou, Z., Lopez, D. L., Kanost, M. R., Evans, J. D. and Jiang, H. (2006). Comparative analysis of serine protease-related genes in the honey bee genome: possible involvement in embryonic development and innate immunity. *Insect Mol. Biol.* **15**, 603-614.