Journal of Apicultural Research 53(5): 576-589 (2014)
DOI 10.3896/IBRA.1.53.5.11

ORIGINAL RESEARCH ARTICLE

Apis mellifera haemocytes *in-vitro*: What type of cells are they? Functional analysis before and after pupal metamorphosis

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Received 5 December 2012, accepted subject to revision 24 October 2013, accepted for publication 12 December 2013.

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Summary

The honey bee *Apis mellifera* is affected by many parasites posing a serious threat to our ecosystem, agriculture and apiculture. The study of *A. mellifera* immune response represents a great complement for developing integrated disease management strategies. This work presents updated data concerning the *in vitro* cellular immune response of *A. mellifera* fifth instar larvae (L5) and newly emerged workers (W). Haemocytes from *A. mellifera* L5 and W clearly displayed different appearance *in-vitro*. The blood cells from these two developmental stages yielded behavioural differences regarding attachment and spreading. However, haemocytes extracted from L5 and W exhibited strong similarities with respect to their general (multicellular) behavioural pattern. This multi-haemolytic process, triggered after glass contact, could result from an encapsulation-like response. At the same time, an experimental system for studying *A. mellifera* cellular immunity *in-vitro* upon recognition of glass surfaces and haemolytic encapsulation response is herein developed.

Hemocitos de *Apis mellifera in vitro*: ¿qué tipo de células son? Análisis functional antes y después de la metamorphosis de la pupa

Resumen

La abeja melífera *Apis mellifera* se ve afectada por muchos parásitos que suponen una grave amenaza para nuestro ecosistema, la agricultura y la apicultura. Estudiar el sistema inmunológico de *A. mellifera* aportaría información de gran relevancia para el desarrollo de estrategias de manejo integrado de plagas. Hasta el momento, no contamos con datos actualizados relativos a la defensa inmune celular "hemocítica" de *A. mellifera.* En este trabajo estudiamos *in-vitro* aspectos relacionados a la inmunidad celular de larvas del quinto estadío (L5) y obreras recién emergidas (W) de *A. mellifera*. Los hemocitos de L5 y W mostraron claras diferencias respecto a la apariencia, adherencia y esparcimiento (fenotipo de activación inmunológica celular) *in-vitro*. Sin embargo los hemocitos de L5 y W exhibieron un patrón de comportamiento multicelular general asociado a una respuesta de encapsulación disparada después del contacto con una superficie de vidrio *in-vitro*. De esta forma, en este trabajo describimos un sistema experimental fiable para realizar experimentos *in-vitro* para ahondar en el estudio de la aptitud inmune celular de *A. mellifera*, desde las primeras etapas del reconocimiento de lo "no propio" mediado por los hemocitos hasta respuestas multicelulares relacionadas a la encapsulación.

Keywords: *Apis mellifera,* immunity, haemocytes, *in-vitro*

Introduction

Worldwide losses of honey bee colonies have raised genuine public concern (Potts *et al.*, 2010; Levy, 2011). As global decline continues for many other social and solitary bees, the deterioration of insect-mediated pollination may critically affect agricultural and natural ecosystems (Potts *et al.*, 2010). In recent years, researchers have reported numerous possible explanations for honey bee disappearance (Potts *et al.*, 2010; Hendriksma *et al.*, 2011). In the United States, a strange phenomenon known as colony collapse disorder (CCD) or honey bee depopulation syndrome (HBDS) has been reported (Levy, 2011). Habitat loss, pesticide poisoning,

microparasites, bacteria and parasitic mites, in whole or in part, may lie behind the mysterious syndrome (Drysdale *et al.*, 2007; Vanengelsdorp *et al.*, 2009; Levy, 2011). As a consequence, loss of *A. mellifera* colonies represents a serious threat to the ecosystem, agriculture and apiculture (Levy, 2011). Among the parasites affecting bee health, the parasitic mite *Varroa destructor* is to be considered, having been identified as responsible extensive honey bee colony losses (Neumann and Carreck, 2010; Martin *et al.*, 2012).

Another serious threat to *A. mellifera* is American foulbrood (a severe bacterial disease affecting bee larvae) caused by *Paenibacillus larvae;* and the emergent microparasite *Nosema ceranae* (a fungus which affects bee gut). All of these parasites and pathogens drastically affect honey bees producing colony disease (Potts *et al.*, 2010).

To defend themselves against parasitic and infectious organisms, insects have a well-developed innate immune system (Strand, 2008). Therefore, studying the *A. mellifera* immune system could provide basic information to better understand how honey bees defend themselves against parasites. In agreement with this, the effects of pesticides and parasites on *A. mellifera* immune system, development, detoxification responses and colony level have become key topics that need to be addressed (Boncristiani *et al.*, 2012; Dietemann *et al.*, 2012).

Innate immune defence comprises humoral and cellular responses. Humoral response refers to soluble effector molecules such as antimicrobial peptides, complement-like proteins, and enzymatic cascades that regulate melanin formation and clotting. Cellular immunity consists in cell-mediated responses like phagocytosis, encapsulation and wound closure (Strand, 2008). The elimination of organisms entering the insect haemocoel requires that blood cells (haemocytes) be able to recognize and respond to the invading species (Clark *et al.*, 1997). When recognizing a surface as foreign, haemocytes quickly transform from resting non-adherent cells to activated adherent cells that first adhere as a monolayer or as foci to the foreign surface (Nardi *et al.*, 2006). After non-self recognition, the response of the insect haemocytes is to 'spread' (Gillespie *et al.*, 2006). If the foreign agent is small, this spreading ability promotes particle phagocytosis, whereas larger foreign objects (or many small foreign objects together) would be subject to nodulation or encapsulation owing to the cooperative action of a number of haemocytes (Eleftherianos *et al.*, 2009). In most insects, multicellular parasites and other foreign targets entering the haemocoel are eliminated by cellular encapsulation (Strand, 2008). In this sense, inhibiting haemocyte spreading is a common strategy adopted by many entomopathogens (Dean *et al.*, 2004). This implies that such changes in haemocyte behaviour are important components of insect defence system (Eleftherianos *et al.*, 2009).

With respect to cellular insect defence, most literature is focused on model species like *Drosophila melanogaster*, selected Lepidoptera and mosquitoes (Strand, 2008). Unfortunately data concerning haemocyte characterization from Hymenoptera are scarce.

Apis mellifera represents an ideal organism to investigate ontogenetic changes in the immune system for combining holometabolous development within a eusocial caste system (Laughton *et al.*, 2011). Holometabolous insects suffer drastic morphological and physiological changes throughout their development. In line with this, the pattern of the cellular elements in the larval haemolymph could be quite different from that present in the adult (Manfredini *et al.*, 2008). Interesting changes in the immune aptitude of *A. mellifera* during ontogeny have been accounted for (Wilson-Rich *et al.*, 2008; Laughton *et al.*, 2011). Nevertheless, most research efforts to date centre on humoral immune response and much less on honey bee cellular defence (Gätschenberger *et al.*, 2013; Bedick *et al.*, 2001; Evans, 2006; Wilson-Rich *et al.*, 2008). On the contrary, most of our knowledge about cellular immunity of honey bees is based on a few discontinued works dealing with honey bee haemocyte characterization and the effects of varroa parasitism on *A. mellifera* blood cells (Price and Ratcliffe, 1974; Fluri *et al.*, 1977; Van Steenkiste *et al.*, 1988; Wienand and Madel, 1988; Beisser *et al.*, 1990; de Graaf *et al.*, 2002; Sapcaliu *et al.*, 2009). Indeed, it is imperative to delve deeper into the cellular immune response while exploring bee defence against parasites and pathogens.

The study of insect haemocytes has historically been controversial since comparisons are often based on different methodological viewpoints (Ribeiro and Brehélin, 2006). The high reactivity of insect haemocytes does not allow to compare the results obtained from different experimental systems. In general, when comparisons are based only on morphological characterization confusion is created. The best quality information has been obtained from a functional comparison of haemocytes from Lepidoptera, *Drosophila* and mosquitoes (Ribeiro and Brehélin, 2006; Strand, 2008).

The main purpose of this work was to study *A. mellifera* haemolytic behaviour *in-vitro*. Previous works have put forward the relevance of the functional analysis of honey bee haemocytes *in-vitro* (Negri *et al.*, 2013). In this case, methodologies to perform *in-vitro* experiments with *A. mellifera* haemocytes are proposed, some of them backed up by the ones reported by Negri *et al* (2013). This study focuses on describing the in-vitro behaviour of haemocytes from two developmental stages of *A. mellifera*: Fifth instar larvae and newly emerged adults. We hypothesized that fifth instar larvae and worker haemocytes present morphological differences between them, based on previous studies that described changes in the immune aptitude during *A. mellifera* ontogeny (Wilson-Rich *et al.*, 2008; Laughton *et al.*, 2011; Gätschenberger *et al.*, 2013).

Material and methods

Insects

Apis mellifera brood combs were collected from the J J Nagera experimental apiary which belongs to the Arthropods Laboratory of the Universidad Nacional de Mar del Plata located in Ruta Nº 11 (38º10´06´´S; 57º38´10´´W). Combs were cleaned off from workers and taken into an incubator. Two developmental bee stages were used in the experiments. Fifth instar larvae and newly emerged workers were collected immediately upon emergence at 30-32ºC and 70 % relative humidity in the incubator. These two developmental stages were chosen because fifth instar larvae is the main developmental stage insulted by *V. destructor* parasitosis during the reproductive phase of the mite's life cycle. *V. destructor* also parasites adult bees during their phoretic phase, and this explains why we chose an adult bee stage to work with. Newly emerged adults constitute a way of determining and standardising the age of adult honey bees, and permit to study cellular immune response to conduct future comparative studies. Moreover, obtaining haemolymph from newly emerged workers is easier than from adult bees.

Fifth instar larvae were identified based on the size of the larvae, selecting mobile larvae that filled a cell comb completely. The larvae collected from open cell combs corresponded to the beginning of the fifth instar, whereas the larvae collected from closed cell combs corresponded to the end of the fifth instar (Silva-Zacarin *et al.*, 2007). The larvae collected before capping, which corresponded to the beginning of the fifth instar, were used in this case.

Haemolymph collection

Haemolymph collection was performed as previously described by Negri and co-workers (2013). Insect blood was collected from fifth instar larvae by puncturing the soft cuticle with a sterile 30G needle. Workers were punctured in the neck at the aorta and haemolymph was collected from the resulting bubble. Modified plastic tubes were used to immobilize the adult honey bee and expose the aorta. The haemolymph was collected with a micropipette and transferred into a microcentrifuge tube containing ice-cold sterile anticoagulant buffer (AB) (0.098 M NaOH, 0.186 M NaC1, 0.017 M EDTA and 0.041 citric acid, pH 4.5) (Pech and Strand, 1995), phosphate buffered saline (PBS, pH= 7,2) or culture media. Three culture media were utilized: Grace's Insect Media (GIM, Hyclone), TC-100 insect cell culture medium (Sigma) or Excell-405 culture medium (Sigma) (1:10 ratio of haemolymph:PBS/medium). Haemolymph solution was homogenized by soft pipeting into the sampling tube and then transferred to 96 sterile well glass bottom plates (NUNC, 96-well optical bottom plates, sterile). When no adherent haemocytes were transferred to another well, the remaining adherent cells were covered again with buffer or media. Anticoagulant buffer was used when haemocyte clumping, coagulation and melanisation wanted to be avoided. Preliminary experiments were performed using reduced glutathione as a melanisation inhibitor in order to link the pigmentation observed in our experimental system with melanin. When the naive *in-vitro* behaviour of haemocytes interacting with coagulation and melanisation wanted to be analysed the haemolymph was diluted with ice cold PBS or culture media.

Haemocytes immune stimulation with bacterial elicitors

Upon recognizing a surface as foreign, haemocytes transform from resting cells to activated adherent cells that adhere and spread over the foreign surface (Gillespie and Kanost, 2006; Eleftherianos *et al.*,

Table 1. Summary of the characteristics observed for each haemocyte type from the two developmental stages studied shown in Fig. 1.

Stage	Hemocyte Type	Glass adherence	Spread	Granules	Locomotion	Filopods	Pseudopods	Lamellipodia
Fifth Instar larvae (L5)	1	Strong	Yes	Yes	No	No	No	Yes
	2	Smoth-strong	Yes	After 24hs	Yes (active)	No	Yes	Yes
Newly Emerged Worker (W)	1	Strong	Yes	Yes	No	Yes	Yes (short)	No
	2	Strong	Yes	No	No	Yes	Yes	No
	3	No	No	No	Yes (Passive)	No	No	No
	4	No	No	No	Yes (Passive)	Yes	No	No

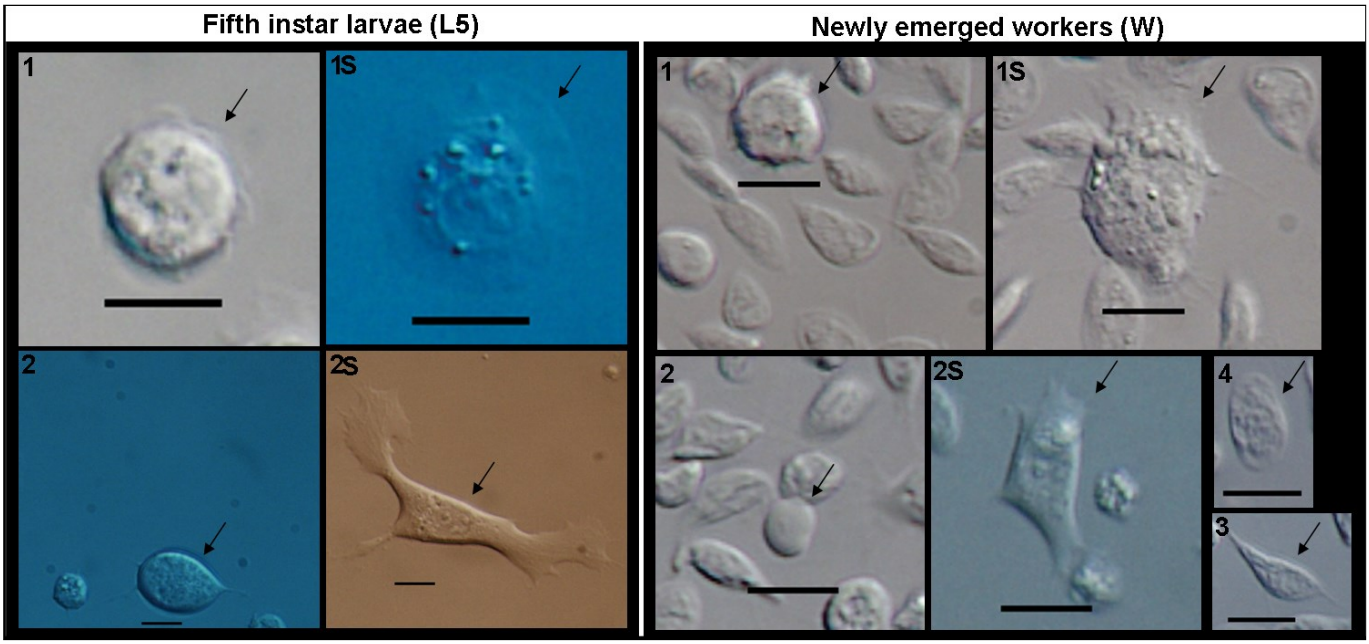

Fig 1. *Apis mellifera* haemocytes observed with DIC microscopy *in-vitro*. Haemocytes from the fifth instar larvae (L5, upper-left panel). Hemocyte types are designated with numbers 1 and 2. Newly emerged worker haemocytes (W, upper-right panel). Hemocyte types are designated with numbers 1 to 4. The letter "S" refers to the spread appearance of each haemocyte type. Scale bar = 10 micrometres.

2009). If the foreign agent is small, spreading results in the phagocytosis of the particle, whereas if it is larger, it would be subject to nodulation or encapsulation owing to the cooperative action of a number of haemocytes (Davies and Preston, 1986). Biotic microbial and non-microbial surfaces as well as abiotic surfaces such as plastic and glass are recognized as non-self by the insect innate immune system. Thus, spreading of haemocytes on a glass surface is a common measure of immune activation (Dean *et al.*, 2004; Negri *et al.*, 2013). Cells that spread upon stimulation with Lipopolysacharide 100 µg/ml (LPS, Sigma), *Paenibacillus larvae* and/or contact with a glass surface were considered immune-related haemocytes. Half volume from each haemolymph containing solution was deposited with an equal volume of PBS/media alone or PBS/media containing different concentrations of the bacterial elicitor or *Paenibacillus larvae*. The same haemolymph sample was equally distributed between each treatment so as to perform the experiments under standardised conditions regarding haemocytes number. This strategy ensured a reduced manipulation of haemocytes because haemocyte count before the experiments was avoided. It also allowed to obtain more individualised haemocytes and hence perform better quantification of the cellular areas; yet the number of haemocytes per well was diminished. Only the haemocytes capable of being individualised were randomly photographed and then measured. Three bacterial challenge experiments were conducted independently; and the haemocytes measured per treatment ranged from 15 to 20. The total cellular area for each haemocyte was measured for cellular spreading quantification using *imageJ* software (Negri *et al.*, 2013).

Encapsulation/nodulation assay

The methods described by Pech and Strand (1995) and Ling and Yu (2005) were combined and modified resulting in the methodology described by Negri and co-workers (Negri *et al.*, 2013). In order to avoid haemocyte adherence, the glass surface of the plate bottom was covered with 30 µl of 1% agarose. This provided a thin layer of agarose that allowed keeping haemocytes in suspension and visualizing them in real time. Many different time points could be performed without the need of several fixation and washing steps. To stimulate nodulation, a 10 microlitre micropipette tip was first introduced into an *Escherichia coli* suspension and then used to touch the centre of the thin layer of agarose before the haemocyte suspension was added. To stimulate encapsulation, synthetic beads (Dowex) were added to the wells.

Haemocyte classification

Standardizing haemolytic classification by comparing previous literature on *A. mellifera* haemocytes description (Price and Ratcliffe, 1974; Fluri *et al.*, 1977; Van Steenkiste *et al.*, 1988; Wienand and Madel, 1988; Beisser *et al.*, 1990; de Graaf *et al.*, 2002; Sapcaliu *et al.*, 2009) turned out to be really tough. As a result, it was decided to differentiate, in the first place, the *A. mellifera* haemocytes studied in this work under the experimental system adopted herein. The chosen criterion was based on parameters such as attachment to glass surfaces, spreading, presence of granules, development of filopodia, or pseudopodia or lamellipodia and locomotion. These are some of the most widely used parameters when it comes to describing

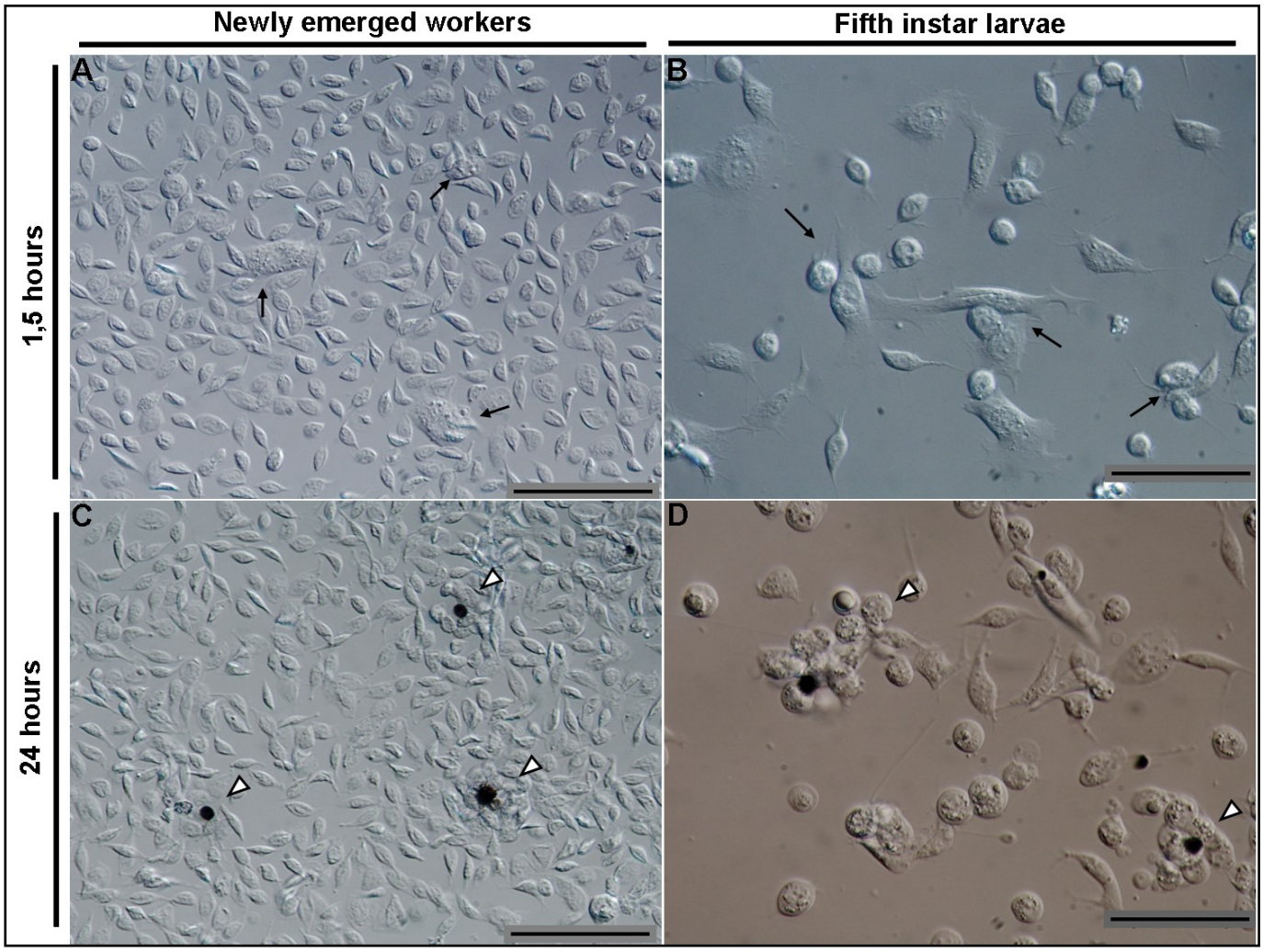

Fig 2. *Apis mellifera* haemocytes cultured *in vitro* for 24 hours. Haemocytes from fifth instar larvae (A,C) and newly emerged workers (B,D) after 1,5 hours (A,B) and after 24 hours (C,D) of culture. Scale bars = 50 micrometres.

haemocytes using similar experimental systems to and observed under the same type of microscopy (DIC) as one of the most studied insects, i.e., Lepidoptera (Ribeiro and Brehélin, 2006; Strand, 2008). The nomenclature adopted resulted from the combination of the developmental stage ("L5" for fifth instar larvae and "W" for newly emerged workers) and the type of haemocyte designated by numbers (1 and 2 for L5 and 1, 2, 3 and 4 for W, respectively). Finally, the functional comparison between *A. mellifera* haemocytes with Lepidoptera was discussed and *A. mellifera* haemocytes denomination using the classic nomenclature for Lepidoptera was proposed.

Fifth instar larvae haemocytes pseudopodia orientation

Haemocytes from the L5 stage showing large pseudopodia were analysed in relation to the location of other haemocyte types observed *in-vitro* when cultured directly in culture media. The association between haemocytes showing pseudopodia oriented towards other haemocyte type location and haemocytes with undefined pseudopodia orientation was measured. A total of 315 haemocytes were analysed.

Bacterial strains

Paenibacillus larvae (*P. larvae*) and *Escherichia coli* (*E. coli*) cultures were courtesy of Dr Liesel Gende and Dr Claudia Studdert research groups from the Universidad Nacional de Mar del Plata, Argentina, respectively. Bacteria were washed and resuspended in the culture media used to sample the haemolymph in each experiment before bacterial challenge.

Microscopy

After being transferred to the 96-well plates, the mixtures containing *A. mellifera* haemolymph were observed for 24 hours using inverted microscopy. Haemocytes were observed by means of differential interference contrast (DIC) microscopy and time lapse imaging. The microscope used was a Nikon Eclipse *Ti* inverted microscope using a 60x objective. For time-lapse imaging, pictures from the same field were taken every minute.

Statistics

The T-test was performed for normally distributed data, while the Mann-Whitney test was utilised for not normally distributed data.

Results

Haemocytes behaviour *in-vitro*

Individual behaviour of each haemocyte type

Different haemocytes were found in *Apis mellifera* haemolymph from the two developmental stages under study (L5 and W). These were described according to morphological and behavioural aspects upon contact with glass, and visualised using DIC microscopy. Afterwards they were designated with a number (Fig. 1 1, Table 1).

L5-1: First fifth instar larvae haemocyte type that strongly attached to and spread over the wells glass bottom. This haemocyte modified its spreading behaviour, but showed no locomotion during the time observed in this work (supplementary material, time lapse imaging 1). Its shape was rounded when it first adhered and rounded with symmetric flattening and lamellipodia surrounding when it spread (Fig. 1 1, table 1, 1S and L5-1, respectively). Granules were also noticeable during spreading (Fig. 1 1, Supplementary Material time lapse imaging 1).

W-1: First worker haemocyte type that strongly attached to and spread over the wells glass bottom. This haemocyte showed extreme spreading behaviour associated to granules formation. Its shape was rounded-oval when it first attached to the glass surface (Fig. 1 1, Table 1, Supplementary Material, time lapse imaging 2) and pleomorphic showing filopodia, lamellipodia and short pseudopodia-related structures when it spread (Fig. 1 1, Table 1, 1S and W-1 respectively).

L5-2: The second fifth instar larvae haemocyte type that spread after glass contact. It showed smooth adherence to the glass bottom. Its shape was rounded-oval when unspread. The extreme pseudopodia development during spreading (Fig. 1 1; Table 1, 2S and L5-2 respectively) was directly associated with notorious locomotion over the glass surface (supplementary material time lapse imaging 1).

W-2: This worker haemocyte type adhered and spread over the glass surface after W-1. Its shape was rounded-oval when it had just attached and pleomorphic upon spreading. These haemocytes featured short pseudopodia development and a smooth membrane with no granules (Fig. 1 1; Table 1, 2S and W-2, respectively).

W-3: This haemocyte type flowed in suspension (supplementary material, time lapse imaging 2) and showed oval flattened appearance (Fig. 1 1; Table 1, W-3).

W-4: This haemocyte type presented spindle shaped morphology, and developed fine filopodia in its ends following the haemocyte longest axis (Fig. 1 1; Table 1, W-4). W-4 flowed in suspension and did not adhere to the glass surface *per-se* (Supplementary Material time lapse imaging 2).

Multicellular behaviour

Apis mellifera haemocytes were sampled directly in PBS or culture media in ice cold conditions (without the use of anticoagulant buffer) and incubated in the wells of the 96-well glass bottom plates. No melanisation or coagulation-inhibiting condition was used for these experiments in order to analyse the naive behaviour of haemocytes *in-vitro*. Under these conditions, haemocytes from *A. mellifera* L5 and W yielded a similar multicellular behavioural pattern evidenced as foci of agglomerating cells (Fig. 1 2). The behavioural pattern described by *A. mellifera* blood cells under these conditions could be divided into three phases, as described below:

First phase: the haemocyte type first adheres to and spreads over the glass surface at the bottom of the well, L5-1 and W-1 (Fig. 1 3).

Second phase: the strongly adherent L5-1 and W-1 are subsequently surrounded by L5-2 or W-3 and 4, respectively (Fig. 1 3).

Third phase: the process ends with foci of cell agglomerations with pigmented cores (Fig. 1 3), and cells exhibiting an apoptosis-associated morphology (Fig. 1 3).

Immune activation of *A. mellifera* haemocytes *in-vitro*

In order to correlate the spreading behaviour of *A. mellifera* haemocytes observed upon glass surface contact with immune activation, L5 haemocytes were challenged with the bacterial elicitor LPS and the bacteria *Paenibacillus larvae.* A tendency towards augmented spreading was observed in L5 haemocytes stimulated with the bacterial challenges (Fig. 1 4) in all the experiments conducted. However, L5-2 spreading behaviour upon *P. larvae* challenge was the only one to become significantly enhanced (Fig. 1 4 B). When incubated with *P. larvae*, L5-1 and 2 showed enhanced spreading morphology (Fig. 1 4) although only the L5-1 type was noticed to have attached-phagocytized-like bacteria (Fig. 1 4 D). Worker haemocytes also showed enhanced spreading when challenged with microbial elicitor and when incubated with *P. larvae*, extracellular matrix and membrane like structures formed in the cultures that appeared to immobilize bacteria (data not shown).

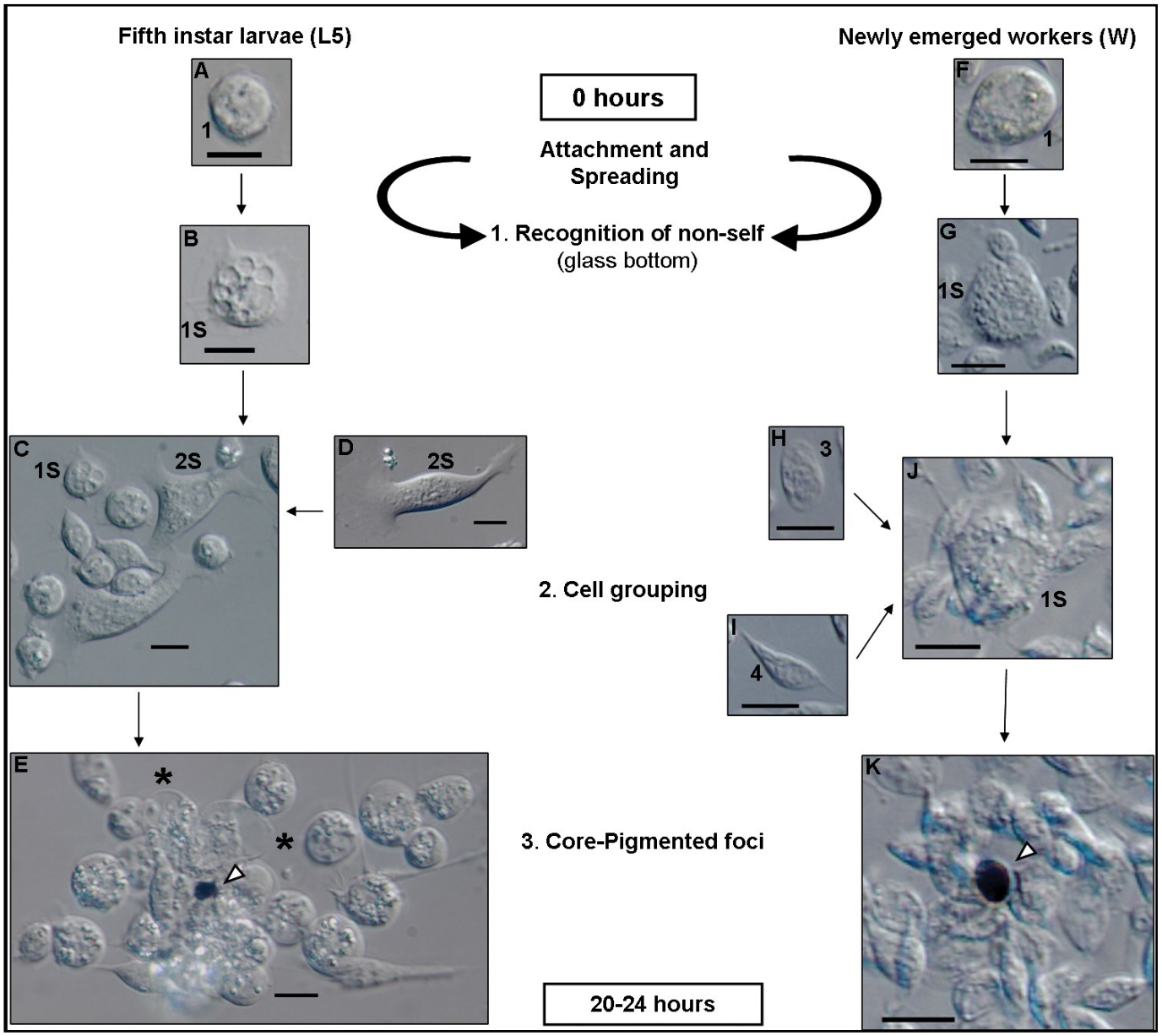

Fig 3. Model for the multicellular response of *Apis mellifera* haemocytes after glass surface contact. Response divided into phases 1, 2, and 3 (centre). Fifth instar larvae (L5, left). Newly emerged worker (W, right). Adherent unspread L5-1 haemocyte type (1, A). Adherent activated (spread) L5-1 (1S, B). Smoothly adherent, spread, migrating L5-2 (2S, D). Foci of agglomerated haemocytes (C and J). Agglomeration of haemocytes after 24 hours of *in-vitro* culture (E and K). Pigmented cores (white arrowheads). Apoptosis associated blebs (asterisks). Strongly adherent W-1 haemocyte type (1, F). Spread W-1 (1S, G). Flowing W-3 (3, H). Flowing W-4 (4, I). Scale bars = 10 micrometres.

L5-2 pseudopodia in relation to L5-1 position

The fifth instar larvae haemocyte type 2 presented extreme pseudopodia development when spread, while the fifth instar larvae pseudopodia showed to be functional to active locomotion over the glass surface (Supplementary Material, time lapse imaging 1). In order to determine a possible pattern for L5-2 direction, the orientation of the longest pseudopodia axis of each L5-2 haemocytes was analysed. Around 78 % of the L5-2 showed pseudopodia oriented towards L5-1 haemocytes positioning (Fig. 1 5). When L5-2 pseudopodia ends were found to be in contact with an L5-1 haemocyte, the latter featured apoptotic appearance (Fig. 1 5).

Worker haemocytes agglomeration is related to non-self recognition

Non-adherent W-3 and 4 haemocytes flowed in suspension showing no active locomotion, but were found to form cell agglomerates on W-1. When cultured in anticoagulant buffer, the non-adherent haemocytes W-3 and 4 did not attach to the strongly adherent granulocytes (nor spread under these conditions), but still appeared near the W-1 and surrounded them (data not shown). In order to associate W-3 and 4 agglomeration related to W-1 location with an immune multicellular interaction upon stimulation with an abiotic non-self surface (glass) of the wells bottom, *in-vitro* nodulation/

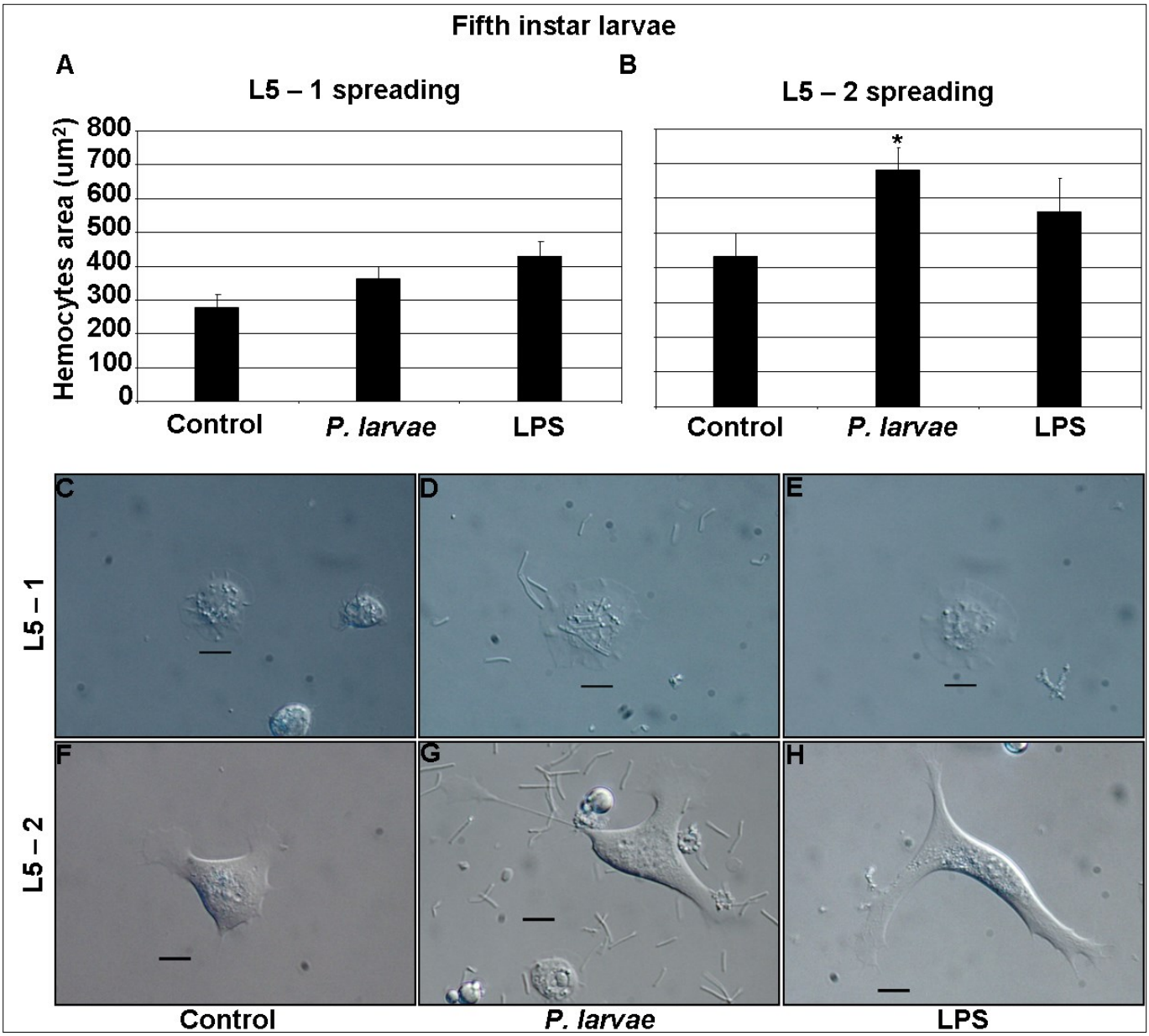

Fig 4. *Apis mellifera* haemocyte spreading after *Paenibacillus larvae* and lipopolysacharide challenge. Fifth instar larvae haemocytes were treated with culture media alone (Control) or culture media plus *Paenibacillus larvae* (*P. larvae*) or culture media plus lipopolysaccharide 100ug/ml (LPS). Then, L5-1 (A, C, D, E) and L5-2 (B, F, G, H) were photographed and their area was measured using *imageJ* software (A,B). Scale bars = 10 micrometers. Asterisk means significant differences with respect to control. T-test p=0,018.

encapsulation experiments were performed. In these experiments, haemocytes agglomerations were located in relation to the large immune stimuli utilised to elicit multicellular responses, like nodulation or encapsulation (Fig. 1 6 E, F). When no immune challenge was added to the agarose coated wells, haemocytes flowed freely and showed no adherent haemocyte type after 24 h (Fig. 1 6 D). Noticeably, haemolytic attachment to agarose could be followed in the well, reflecting the circular shape of the micropipette tip used to seed the bacteria (Fig. 1 6 F). Also, when observed in the chromatographic beads used for the encapsulation assay, the foci of agglomerated haemocytes could be recognized. Moreover, the synthetic beads were surrounded by haemocytes (Fig. 1 6 F). The attachment capability after 24 hours reflected haemocytes wellbeing under the experimental conditions, and evidenced the relationship existing between adherence and non-self recognition.

Discussion

New methodical development is in urgent need to address the factors underlying globally observed bee losses. Advances in this subject are necessary for the development of field, semi-field and laboratory standard testing methods (Hendriksma *et al.*, 2011; Dietemann *et al.*, 2012). In this sense, a lot of work is being directed at the standardization of laboratory protocols in order to reduce undesirable results variability worldwide (Hendriksma *et al.*, 2011). So far *A.*

mellifera cellular immune strength has been mostly indirectly measured and associated with the number of haemocytes counted from haemolymph (Wilson-Rich et al., 2008) or the number of nodules formed (Gätschenberger *et al.*, 2013). New methodology for honey bee cellular immune aptitude screening is herein described.

Indeed the study of insect haemocytes has always been challenging. Haemocytes are very reactive and labile cells that quickly activate after haemolymph extraction making *in-vitro* study difficult (Markus *et al.*, 2005). The classification of insect haemocytes could be subject to controversy, and the terminology employed to designate each cellular type often differs from one species to another (Ribeiro and Brehélin, 2006). Small differences between the *in-vitro* methods used could lead to different results and hence to controversial interpretations (Tojo *et al.*, 2000; Ribeiro and Brehélin, 2006). In this sense, choosing the experimental system as the first step in the study of an insect cellular defence seems to be a proper decision.

In regard to insect cellular defence, most literature focuses on model species like *Drosophila melanogaster*, selected Lepidoptera and mosquitoes (Ribeiro and Brehélin, 2006; Strand, 2008). When comparing the haemocytes from *Drosophila*, Lepidoptera and mosquitoes the morphological descriptions do not necessarily coincide. Interestingly, the functional and behavioural comparisons between these insect haemocytes have contributed with relevant information and complemented morphologic criteria (Ribeiro and Brehélin, 2006). The importance of performing functional and behavioural description of haemocytes *in-vitro* has been very well reflected by Dr Michael Strand and co-workers in *Pseudoplusia includens* (Pech and Strand, 1995, 1996, 2000; Clark *et al.*, 1997; Lavine and Strand, 2001).

The scarce information available about the cellular defence of *A. mellifera* in these days represents the opportunity to start from the beginning and discuss about the experimental system that best fits

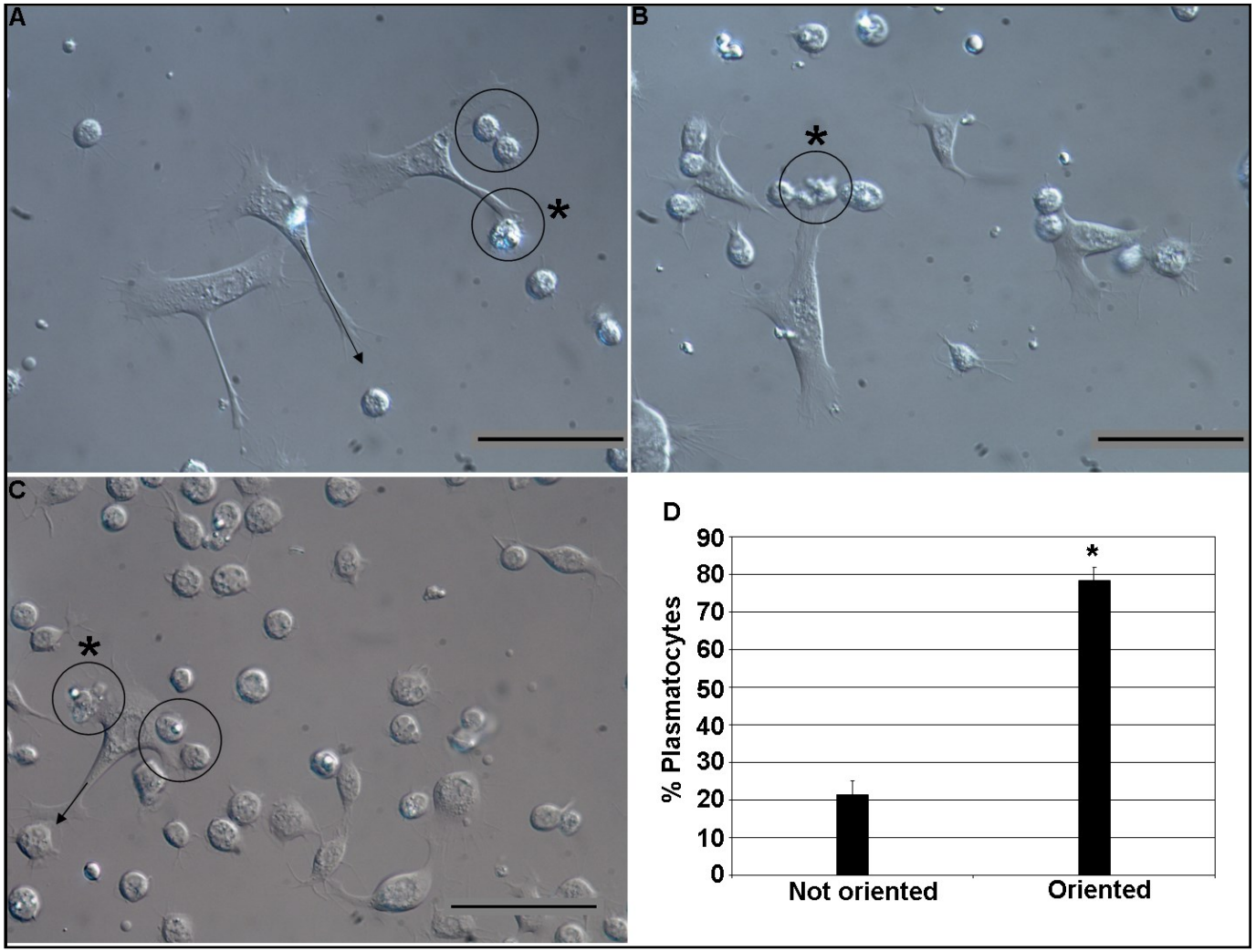

Fig 5. Fifth instar larvae haemocytes type 2 (L5-2) pseudopodia develop in direction to haemocyte type 1 (L5-1) location. Pseudopodia with the longest axis direction (arrows in A, and C), L5-2:L5-1 contacts (circles in A, B and C) and L5-1 apoptosis-like blebs associated with L5-2 contacts (asterisks in A, B and C). D. Percentage of L5-2 with oriented pseudopodia in relation to L5-1 location (Oriented) Vs. Percentage of L5-2 not oriented to L5-1 (Not oriented). A total of 315 L5-2 haemocytes were analysed. Scale bars = 50 micrometres. Asterisk means significant differences between the two groups studied. Mann-Whitney, p = < 0,001.

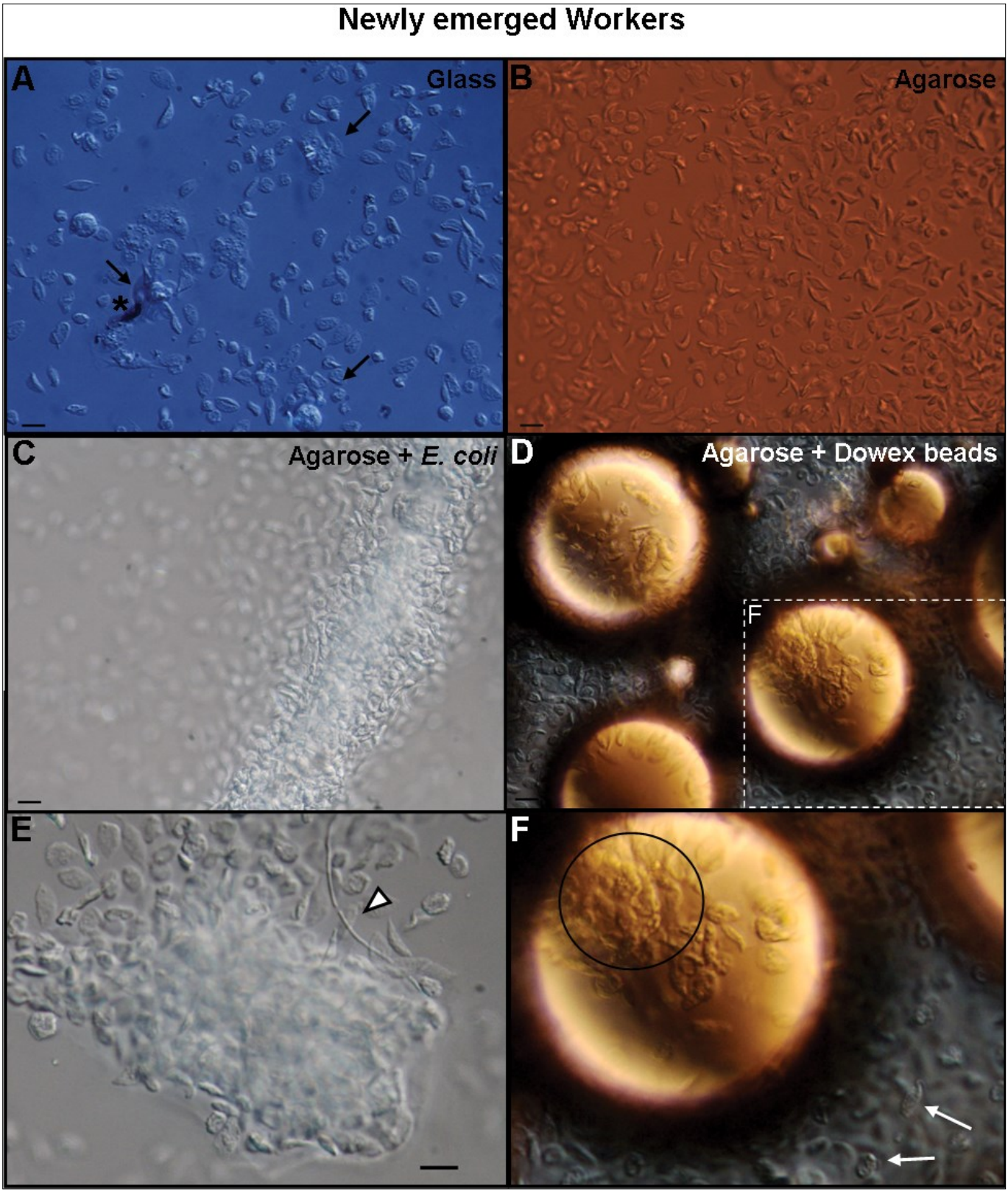

Fig 6. *Apis mellifera* newly emerged worker haemocytes agglomerations are associated with multicellular immune responses. Haemocytes cultured over naked glass surface (A) or over 1% agarose coated glass bottom alone (B). Haemocytes cultured over 1% agarose coated bottom plus chromatography beads (D). F: enlarged detail from picture D delimited for the white dotted line square. C and E: haemocytes cultured in buffer saline over 1 % agarose coating with seeded *Escherichia coli* into the centre using a micropipette tip. Black line circle = agglomeration of haemocytes attached to the beads. Black arrows = melanised coagulation fibres. White arrows = flowing W-4 haemocytes. Scale bars = 10 micrometres.

researchers inquires. Complementing the morphological characterization of *A. mellifera* haemocytes with functional descriptions *in-vitro* is a must.

The immune activation after the non-self recognition of insect haemocytes is evidenced by the attachment to and spreading over the foreign surface. Spreading over glass surfaces by insect haemocytes is used as an immune fitness measure (Dean *et al.*, 2004). Nonetheless the physiological state of both the cell and the organism may condition haemocytes morphology and behaviour (Manfredini *et al.*, 2008). In this sense, regarding insects subject to drastic morphological and physiological changes throughout their development, the cellular elements in the larval haemolymph may differ significantly from those present in the adult (Manfredini *et al.*, 2008).

In concordance with the description *ut supra*, the methodology proposed in this work allowed to study *A. mellifera* haemocytes immune activation (spreading) over time (up to 24 hours) after immune stimulation in biotic (*P. larvae*, *E. coli*, LPS) or abiotic non-self (glass) surfaces. Two developmental stages were studied in this work: fifth instar larvae (L5) and newly emerged workers (W). These are the ontogenic stages of *A. mellifera* before and after pupae metamorphosis, respectively.

The haemocytes extracted from *A. mellifera* L5 and W showed noticeable differences regarding morphology and behaviour. Clearly, the metamorphosis between these two ontogenic stages played a key part on *A. mellifera* cellular immune defence. Haemocytes from L5 and W displayed behavioural differences when analysed on an individual basis, but behaved functionally similarly during the cooperative multicellular response on glass surfaces. Workers haemocytes (W-3 and W-4) did not adhere to glass and presented no active locomotion in suspension. Yet these haemocyte types attached to activated (spread) glass adherent haemocyte W-1. In addition, W-3 and W-4 were always negative to trypan blue staining (data not shown). These evidences reveal that W-3 and W-4 are viable in our experimental system. After 20-24 hours, many foci of overlapping cells formed following W-1/3/4 interaction, and pigmented cores appeared in the haemolytic agglomerations. This interaction between worker haemocytes showed to be related to non-self recognition after bacteria or chromatography beads challenges. Fifth instar larvae also featured cellular agglomerations on glass surfaces comprised by L5-1 and L5-2 haemocyte types. After 20 hours, L5-2 appeared agglomerated in relation to L5-1. L5-1 was the first haemocyte type to strongly adhere to glass, followed by L5-2 spreading. The latter showed extreme locomotion-related pseudopodia development when spread. The longest axis of L5-2 pseudopodia was found to be related to L5-1 position. In addition, L5-2 spreading was enhanced after bacterial challenge, thereby indicating that this response is tied to the immune response. Due to the immobile behaviour exhibited by L5-1 upon spreading over glass surfaces, it could be hypothesised that L5-2 could migrate towards L5-1 and lead to the haemocyte agglomerations observed after 20 to 24 hours. More experiments are definitely required to confirm this hypothesis. In addition, after 20 to 24 hours, L5 haemocytes showed to form multicellular foci with pigmented cores, and haemocytes with apoptosis related morphology appeared. In line with that, workers and L5 larvae contained haemocytes with different appearance and behaviour *in-vitro* when analysed individually but developed the same multicellular response on glass surfaces.

In their work with *Pseudoplusia includes,* Pech and Strand (1996) described the sequence by which granulocytes are the first haemocytes to adhere to the foreign target (chromatography beads) and release signals that activate plasmatocytes. This is followed by a sheath of overlapping plasmatocytes and the encapsulation process ends with some granular cells that attach to the plasmatocyte layer. These same authors also reported that plasmatocytes induce granular cells apoptosis (Pech and Strand, 2000). Both granular cells and

Online Supplementary Material. Time Lapse Imaging 1.

Apis mellifera haemocytes extracted from fifth instar larvae (L5). Haemolymph was mixed in PBS 1x and placed in 96-well glass bottom plates and then visualized under an inverted microscope. Images were taken every minute in order to make this animation. The animation was provided to better describe the behaviour of haemocytes extracted from L5. In this sense, notice that L5-2 haemocytes are smoothly adherent haemocytes that show extreme pseudopodia development associated with active locomotion over the glass surface. This animation also shows a spread adherent L5-1 haemocyte with granules (left). L5-1 is attached to the glass surface and did not show locomotion behaviour during the times observed in this work. http://www.ibrabee.org.uk/index.php/component/content/article?layout=edit&id=3729

Online Supplementary Material. Time Lapse Imaging 2.

Apis mellifera haemocytes extracted from newly emerged workers (W). Haemolymph was mixed in PBS 1x and seeded into 96-well glass bottom plates and then visualized under an inverted microscope. Images were taken every minute to make this animation. The animation was provided in order to better describe the behaviour of haemocytes extracted from one-day old nurses. In this sense, notice that W-3 and W-4 haemocytes flow in suspension when alone but adhere to spread W-1 or forming foci. This animation also shows spread and non-spread adherent W-1 and haemocytes agglomeration. http://www.ibrabee.org.uk/index.php/component/content/article?layout=edit&id=3730

plasmatocytes from *Galleria mellonella* avidly adhere to glass slides. This has been related to encapsulation initiation induced by glass surface (Brooks and Dunphy, 2005). Ling and Yu postulated that *Manduca sexta* pro-phenoloxidase (proPO) bound to the surface of granulocytes and that haemocyte surface proPO could be activated *in-vitro*, leading to the melanisation of these haemocytes (2005). In addition, Altincicek *et al* (2008) stated that nucleic acids derived from hosts trigger coagulation and other immune defence mechanisms in *Galleria mellonella*.

Some similarities could be found if the results revealed in this work are compared to the functional analysis of lepidopteran haemocytes. Consequently, it could be hypothesised that *A. mellifera* fifth instar and workers haemocytes look different but behave like lepidopteran blood cells *in-vitro*. Based on this concept, L5-2 and W-3 and W-4 from *A. mellifera* could function as lepidopteran plasmatocytes inducing apoptosis of L5-1 and W-1, respectively. The latter could function as lepidopteran granulocytes. In addition, the pigmented cores could be melanised nuclei caused by haemocyte disruption and/or haemolytic surface proPO activation involved in an encapsulation-related response in *A. mellifera* triggered by the glass surface. Fig. 1 3 illustrates a model for this. However further studies should be conducted in order to confirm this hypothesis.

Nevertheless, a method based on well-incubated haemocytes is proposed in this work as compared to haemocyte monolayers, which is the most commonly used methodology reported for other insects. *Apis mellifera* worker W-3 and W-4 haemocytes did not adhere to glass but over-activated W-1 did, thereby suggesting some kind of interaction between these haemocyte types. *In-vitro* encapsulation/ nodulation allowed correlating the agglomeration on W-1 with a multicellular cooperative immune response. This fact was of great relevance when it came to choosing the experimental system described herein. The interaction between these two types of haemocytes is lost over washing steps when working with haemocytes over coverslips (data not shown), leading to misinterpretations of the results obtained. This is why a method based on haemocytes incubated in wells is proposed in this work: it allowed us to study and compare *in-vitro A. mellifera* workers with L5 haemocytes under the same experimental conditions.

Thanks to this methodology, it was possible to perform more than one observation/quantification of the same experiment diminishing the manipulation of the cells involved in the assay.

The aspects expressed earlier contributed to considering the methodology proposed in this manuscript as a reliable and practical experimental system for assaying the effects of different substances or molecules on *A. mellifera* cellular immune responses *in-vitro* (Negri *et al*., 2013).

Acknowledgements

This research was supported by "ANPCyT", Argentina (PICT Redes 2006 Nº 00890 Nodo 01). The authors would like to thank CONICET and the UNMdP. Pedro Negri is a doctoral fellow from CONICET, Argentina. The authors are very grateful to Dr Michael Strand for valuable advice, to Dr Liesel Gende for the *P. larvae* strain, to Dr Claudia Studdert for *E. coli* strain and to Lic. Leonardo De Feudis for beekeeping support.

References

ALTINCICEK, B; STOTZEL, S; WYGRECKA, M; PREISSNER, K T; VILCINSKAS, A (2008) Host-derived extracellular nucleic acids enhance innate immune responses, induce coagulation, and prolong survival upon infection in insects. *Journal of Immunology*, 181: 2705-2712. http://dx.doi: 10.4049/jimmunol.181.4.2705

BONCRISTIANI, H; UNDERWOOD, R; SCHWARZ, R; EVANS, J D; PETTIS, J (2012). Direct effect of acaricides on pathogen loads and gene expression levels in honey bees *Apis mellifera*. *Journal of Insect Physiology* . http://dx.doi.org/10.1016/j.jinsphys.2011.12.011

BROOKS, C L; DUNPHY, G B (2005) Protein kinase A affects *Galleria mellonella* (Insecta: Lepidoptera) larval haemocyte non-self responses. *Immunology and Cell Biology*, 83: 150–159. http://dx.doi: 10.1111/j.1440-1711.2005.01316.x

BEDICK, J C; TUNAZ, H; ALIZA, A R N; PUTNAM, S M; ELLIS, M D; 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. *Comparative Biochemistry and Physiology*, 130: 107-117. http://dx.doi: 10.1016/S1532-0456(01)00226-5

BEISSER, K; MUNZ, E; REIMANN, M; RENNER-MULLER, I C E (1990) Experimentelle Untersuchungen zur in uitro-Kultivierung von Zellen der Karntner Honigbiene (*Apis mellifera carnica* Pollmann, 1879). *Journal of Veterinary Medicine*, 37: 509-519.

CLARK, K D; PECH, L L; STRAND, M R (1997) Isolation and identification of a plasmatocyte-spreading peptide from the haemolymph of the Lepidopteran insect *Pseudoplusia includens*. *Journal of Biological Chemistry*, 272: 23440–23447. http://dx.doi: 10.1074/jbc.272.37.23440

DE GRAAF, D C; DAUWE, R; WALRAVENS, K; JACOBS, F J (2002) Flow cytometric analysis of lectin-stained haemocytes of the honey bee (*Apis mellifera*). *Apidologie*, 33: 571-579. http://dx.doi: 10.1051/apido:2002041

DEAN, P; RICHARDS, E H; EDWARDS, J P; REYNOLDS, S E; CHARNLEY, K (2004) Microbial infection causes the appearance of haemocytes with extreme spreading ability in monolayers of the tobacco hornworm *Manduca sexta*. *Developmental and Comparative Immunology*, 28: 689–700. http://dx.doi:10.1016/j.dci.2003.11.006

DIETEMANN, V; PFLUGFELDER, J; ANDERSON, D; CHARRIÈRE, J D; CHEJANOVSKY, N; DAINAT, B; DE MIRANDA, J; DELAPLANE, K; DILLIER, F X; FUCH, S; GALLMANN, P; GAUTHIER, L; IMDORF, A; KOENIGER, N; KRALJ, J; MEIKLE, W; PETTIS, J; ROSENKRANZ, P; SAMMATARO, D; SMITH, D; YAÑEZ, O; NEUMANN, P (2012) *Varroa destructor*: research avenues towards sustainable control. *Journal of Apicultural Research,* 51(1): 125-132. http://dx.doi.org/10.3896/IBRA.1.51.1.15

COX-FOSTER, D L; CONLAN, S; HOLMES, E C; PALACIOS, G; EVANS, J D; MORAN, N A; QUAN, P-L; BRIESE, T; HORNIG, M; GEISER, D M; MARTINSON, V; VANENGELSDORP, D; KALKSTEIN,, A L; DRYSDALE, A; HUI, J; ZHAI, J; CUI, L; HUTCHISON, S K; SIMONS, J F; EGHOLM, M; PETTIS, J S; LIPKIN, W I (2007) A metagenomic survey of microbes in honey bee colony collapse disorder. *Science,* 318: 283-287. http://dx.doi.org/10.1126/science.1146498

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 defences against bacterial infection. *Journal of Experimental Biology*, 212: 1840-1848. http://dx.doi: 10.1242/jeb.026278

EVANS, J D (2006) Beepath: An ordered quantitative-PCR array for exploring honey bee immunity and disease. *Journal of Invertebrate Pathology*, 93: 135–139. http://dx.doi: 10.1016/j.jip.2006.04.004

FLURI, P; WILLE, H; GERIG, L; LUSCHER, M (1977) Juvenile hormone, vitellogenin and haemocyte composition in winter honey bees (*Apis mellifera*). *Cellular and Molecular Life Science*, 33: 1240-1241.

GÄTSCHENBERGER H, AZZAMI K, TAUTZ J, BEIER H (2013) Antibacterial immune competence of honey bees (*Apis mellifera*) is adapted to different life stages and environmental risks. *PLoS ONE,* 8(6): e66415. http://dx.doi.org/doi:10.1371/journal.pone.0066415

GILLESPIE, J P; KANOST, M R (2006) Biological mediators of insect immunity. *Annual Reviews of Entomology*, 42: 611–43. http://dx.doi: 10.1146/annurev.ento.42.1.611

HENDRIKSMA, H; HARTEL, S; STEFFAN-DEWENTER, I (2011) Honey bee risk assessment: new approaches for *in-vitro* larvae rearing and data analyses. *Methods in Ecology and Evolution*. http://dx.doi.org/10.1111/j.2041-210X.2011.00099.x

LAUGHTON, A M; BOOTS, M; SIVA-JOTHY, M T (2011) The ontogeny of immunity in the honey bee, *Apis mellifera* L. following an immune challenge. *Journal of Insect Physiology*, 57: 1023–1032. http://dx.doi:10.1016/j.jinsphys.2011.04.020

LAVINE, M D; STRAND, M R (2001) Surface characteristics of foreign targets that elicit an encapsulation response by the moth *Pseudoplusia includens. Journal of Insect Physiology*, 47: 965–974. http://dx.doi:10.1016/S0022-1910(01)00071-3

LEVY, S (2011) What´s best for bees. *Nature*, 479: 164-165. http://dx.doi: 10.1038/479164a

LING, E; YU, X (2005) Prophenoloxidase binds to the surface of haemocytes and is involved in haemocyte melanisation in *Manduca sexta. Insect Biochemistry and Molecular Biology*, 35: 1356–1366.

MANFREDINI, F; DALLAI, R; OTTAVIANI, E (2008) Circulating haemocytes from larvae of the paper wasp *Polistes dominulus* (Hymenoptera, Vespidae). *Tissue and Cell*, 40: 103–112. http://dx.doi:10.1016/j.tice.2007.10.003

MARKUS, R; KURUCZ, E; RUS, F; ANDÓ, I (2005) Sterile wounding is a minimal and sufficient trigger for a cellular immune response in *Drosophila melanogaster*. *Immunology Letters*, 101: 108–111. http://dx.doi:10.1016/j.imlet.2005.03.021

MARTIN, S J; HIGHFIELD, A C; BRETTELL, L; VILLALOBOS, E M; BUDGE, G E; POWELL, M; NIKAIDO, S; SCHROEDER, D C (2012) Global honey bee viral landscape altered by a parasitic mite. *Science*, 336: 1304. http://dx.doi: 10.1126/science.1220941

NARDI, J B; PILAS, B; BEE, M C; ZHUANG, S; GARSHA, K; KANOST, M R (2006) Neuroglian-positive plasmatocytes of *Manduca sexta* and the initiation of haemocyte attachment to foreign surfaces. *Developmental and Comparative Immunology*, 30: 447–462. http://dx.doi:10.1016/j.dci.2005.06.026

NEGRI, P; MAGGI, M; BRASESCO, C; CORREA-ARAGUNDE, N; EGUARAS, M; LAMATTINA, L (2013) Nitric oxide participates at the first steps of *Apis mellifera* cellular immune activation in response to non-self recognition. *Apidologie*, 44: 575–585. http://dx.doi: 10.1007/s13592-013-0207-8

NEUMANN, P, CARRECK, N L (2010) Honey bee colony losses. *Journal of Apicultural Research* 49(1): 1-6. http://dx.doi.org/10.3896/IBRA.1.49.1.01

PECH, L L; STRAND, M R (1995) Encapsulation of foreign targets by haemocytes of the moth *Pseudoplusia inchdens* (Lepidoptera: Noctuidae) involves an RGD-dependent cell adhesion mechanism. *Journal of Insect Physiology*, 41: 481-488.

PECH, L L; STRAND, M R (1996) Granular cells are required for encapsulation of foreign targets by insect haemocytes. *Journal of Cell Science*, 109: 2053-2060.

PECH, L L; STRAND, M R (2000) Plasmatocytes from the moth *Pseudoplusia includens* induce apoptosis of granular cells. *Journal of Insect Physiology*, 46: 1565–1573. http://dx.doi: 10.1016/S0022-1910(00)00083-4

POTTS, S G; BIESMEIJER, J C; KREMEN, C; NEUMANN, P; SCHWEIGER, O; KUNIN, W E (2010) Global pollinator declines: trends, impacts and drivers. *Trends in Ecology and Evolution,* 25: 345-353. http://dx.doi:10.1016/j.tree.2010.01.007

PRICE, C D; RATCLIFFE, A (1974) A reappraisal of insect haemocyte classification by the examination of blood from fifteen insect orders. *Z. Zellforsch*, 147: 537-549.

RIBEIRO, C; BREHÉLIN, M (2006) Insect haemocytes: What type of cell is that? *Journal of Insect Physiology*, 52: 417–429. http://dx.doi: 10.1016/j.jinsphys.2006.01.005

SAPCALIU, A; RĂDOI, I; PAVEL, C; TUDOR, N; CĂUIA, E; SICEANU, A; MEIU, F (2009) Research regarding haemocyte profile from *Apis mellifera carpatica* bee haemolymph originated in the south of Romania. *Lucrări Stiinlifice Medicină Veterinară*, vol. XLII.

SILVA-ZACARIN, E C M; TOMAINO G A; BROCHETO-BRAGA, M R; TABOGA S R; SILVA DE MORAES, R L M (2006) Programmed cell death in the larval salivary glands of *Apis mellifera* (Hymenoptera, Apidae). *Journal of Biosciences* 32: 309–328.

STRAND, M R (2008) The insect cellular immune response. *Insect science*, 15: 1-14. http://dx.doi:10.1111/j.1744-7917.2008.00183.x

TOJO, S; NAGANUMA, F; ARAKAWA, K; YOKOO, S (2000) Involvement of both granular cells and plasmatocytes in phagocytic reactions in the greater wax moth, *Galleria mellonella*. *Journal of Insect Physiology*, 46: 1129–1135. http://dx.doi: 10.1016/S0022-1910(99)00223-1

VAN ENGELSDORP, D; EVANS, J D; SAEGERMAN, C; MULLIN, C; HAUBRIGE, 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. http://dx.doi.org/10.1371/journal.pone.0006481

VAN STEENKISTE, D; RAES, H; JACOBS, F (1988) Haemocytes of adult honey bees (*Apis mellifera*). *Annals de la Societé Royale Zoologique de Belgique*, 118: 234- 235.

WHITTEN, M M A; TEW, I F; LEE, B L; RATCLIFFE, N A (2004) A novel role for an insect apolipoprotein (Apolipophorin III) in β-1,3-glucan pattern recognition and cellular encapsulation reactions. *Journal of Immunology*, 172: 2177-2185.

WIENAND, A; MADEL, G (1988) Haemocytes of the honey bee, *Apis mellifera*, and their changes by Varroatosis (Hymenoptera: Apidae). *Entomologia Generalis*, 14: 81-92.

WILSON-RICH, N; DRES, D T; STARKS, P T (2008) The ontogeny of immunity: development of innate immune strength in the honey bee (*Apis mellifera*). *Journal of Insect Physiology*, 54: 1392–1399. http://dx.doi: 10.1016/j.jinsphys.2008.07.016