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Infections with the sexually transmitted pathogen *Nosema apis* trigger an immune response in the seminal fluid of honey bees (*Apis mellifera*)

Running title: Consequences of *Nosema apis* infections on the seminal fluid proteome

Julia Grassl [1], Yan Peng [2], Barbara Baer-Imhoof [1], Mat Welch [1], A. Harvey Millar [1], Boris Baer [1]*

[1] Centre for Integrative Bee Research (CIBER) and ARC Centre of Excellence in Plant Energy Biology, Bayliss Building, [2]School of Animal Biology The University of Western Australia, Crawley WA 6009, Australia

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ABSTRACT

Honey bee (*Apis mellifera*) males are highly susceptible to infections with the sexually transmitted fungal pathogen *Nosema apis*. However, they are able to suppress this parasite in the ejaculate using immune molecules in the seminal fluid. We predicted that males respond to infections by altering the seminal fluid proteome in order to minimize the risk to sexually transmit the parasite to the queen, and her colony. We used iTRAQ isotopic labelling to compare seminal fluid proteins from infected and non-infected males and found that *N. apis* infections resulted in significant abundance changes in 111 of the 260 seminal fluid proteins quantitated. The largest group of proteins with significantly changed abundances consisted of 15 proteins with well-known immune-related functions, which included two significantly more abundant chitinases in the seminal fluid of infected males. Chitinases were previously hypothesized to be involved in honey bee antifungal activity against *N. apis*. Here we show that infection with *N. apis* triggers a highly specific immune response in the seminal fluid of honey bee males.

Introduction

The societies of ants, bees and wasps consist of related individuals that live in close proximity within their colonies. This key characteristic of eusocial living also offers favourable conditions for parasites and pathogens to establish infections and spread among genetically similar hosts [1]. Insect societies also consist of males, which often have lower immunocompetence and/or are more susceptible to parasites compared to their sister workers and queens [2-4]. Increased disease susceptibility of social insect males was hypothesized to be a consequence of fewer immune alleles

present in haploid males compared to diploid workers and queens [5]. Male immunity might also be reduced as a result of the unique reproductive biology of social insects. Because the queens of ants, bees and wasps never re-mate once they have started to lay eggs [3, 6, 7], males need to produce ejaculates consisting of large numbers of high quality sperm. Reduced male immunity could therefore result from trade-offs with other life history traits [8, 9] such as the physiological costs males face to achieve their exceptional levels of reproductive potential. Independent of the proximate causes that determine reduced immunocompetence in social insect males, their physical presence in colonies seems to carry potential fitness costs if they can spread infections within a colony or act as vectors of pathogen transfer to queens during mating [10-14]. However, immunity of social insect males has received remarkably little scientific attention to this point, and we lack information to what degree the immune system of social insect males is able to respond to infections and minimize risks of vertical and horizontal pathogen transfer.

We used the honey bee *Apis mellifera*, where males are susceptible to a number of different pathogens, including the fungus *Nosema apis* [15, 16]. Infections of this globally widespread parasite occur after bees ingest spores that infect and multiply in midgut epithelial cells [17, 18] and use the host cell's ATP machinery to replicate [19]. Host cells eventually burst open and release a next generation of spores [20]. Infected bees eventually suffer from dysentery [21], which can facilitate spore transmission through faecal smear infections. Apart from horizontal propagation, *N. apis* has also been reported to be sexually transmitted [22], because spores are present in ejaculates of infected males and capable of inducing novel infections in queens after artificial inseminations [22]. Honey bee males are highly susceptible to *N. apis* and infections propagate quickly [15]. Although early infections do not cause significant increases in mortality or reduce sperm viability, chronic infections increase

mortality in older males [15]. Drones defend themselves from infection by innate immune responses. Antimicrobial molecules such as chitinases, a group of proteins that target the cell wall of fungi [23, 24], have previously been found in their seminal fluid [25]. The seminal fluid is able to recognize and kill >80% of *N. apis* spores [26] and in at least two distinctly different ways. Whereas non-protein components in the seminal fluid induce a direct viability loss of intact spores [26]. Immune response proteins induce extracellular spore germination, disrupting the lifecycle of *N. apis*. A proteomic investigation of seminal fluid from uninfected males has revealed the presence of a number of well-characterised immune response proteins such as chitinase, phosphatidylethanolamine-binding protein and cyclophilin [25, 27].

Here, we investigated whether males are able to raise a specific immune response to *N. apis* infections by comparing the seminal fluid proteomes of infected and non-infected males. We predicted that males have an antifungal immune system in their seminal fluid to mitigate the increased risk of transmitting the pathogen to the queen during mating. We wanted to investigate whether immune response proteins that changed in abundances after infection are members of known insect immune pathways, and how they were involved in antifungal activities. To test for the effects of *N. apis* infections on the phenotypic level, we complemented our proteomic data by testing whether the seminal fluid of infected males differed in its efficiency to kill *N. apis* spores or its ability to keep sperm alive.

Experimental Section

Collecting *N. apis* spores

Western Australia remains free of *Nosema ceranae* [28] that only recently switched from its original host, the Asian honey bee (*Apis cerana*) to *A. mellifera* [29] and became widespread in different global honey bee populations [30, 31]. Consequently, we were able to study effects of *N. apis* infections on males in the absence of confounding effects of *N. ceranae*. To collect *N. apis* spores we used a technique developed previously [15, 26, 32]. In brief, we collected honey bee workers at the entrance of hives with confirmed *N. apis* infections and freeze-killed them at -20°C. We dissected and pooled the intestines of 20 workers and mixed them into 1 ml of distilled water. After adding a 3 mm tungsten bead (Qiagen, Australia), we homogenised the intestines by manually shaking each sample for 2 min. Next, we layered 0.5 ml of the homogenate onto 1.5 ml of 100 % Percoll (Sigma-Aldrich, Australia), followed by centrifugation at 18,000 x g for 60 min at 4°C. We washed the pellet containing *N. apis* spores in 1.5 ml DDI water, briefly vortexed the sample and centrifuged it again at 20,700 x g for 5 min at 4°C. After repeating these steps three times, we re-suspended the cleaned spore pellet in 100 µL of DDI water, determined the spore concentration [33] and froze the sample at -80°C. Previous work has shown that *N. apis* spore collection conducted in this way did not result in significant spore mortality [32].

Drone breeding, infection, and seminal fluid collection

In January 2013, we split a fully grown colony of *Apis mellifera ligustica*, housed at the University of Western Australia, consisting of a brood box with 8 frames and a honey box with approximately 50,000 bees into four colonies of equal size, each headed by a mated sister queen. The colonies were left undisturbed for 2 months to recover. From each colony 10 random bees were collected to

verify the colony to be free from *N. apis* infection. To raise males we provided each colony with a single male brood frame. Two days prior to emerging, we removed the capped brood and transferred it to an incubator kept at 33°C and 60% humidity. On the day of emergence, we individually fed 300 males with 10,000 *N. apis* spores in 1 µL of sugar water (infected) and 300 males with 1 µL of sugar water (100% w/v) only (control), painted them according to treatment and released them back into their colonies.

When the males had reached sexual maturity at an age of 12 days [8], we collected ejaculates from infected and un-infected males as described previously [34], generating 4 biological replicates of seminal fluid from *N. apis*-infected and 4 biological replicates of seminal fluid from control males. To initiate ejaculation, we anesthetized males in chloroform and gently squeezed their abdomen between two fingers, until a semen droplet of up to 2 µL appeared at the tip of the endophallus. We collected semen into a pipette tip moistened with Hayes solution (0.15 M NaCl, 1.80 mM $CaCl_2$, 2.68 mM KCl, 1.19 mM $NaHCO_3$, adjusted to pH 8.7 using NaOH).

We used a previously established protocol to separate pooled ejaculate samples into seminal fluid and sperm by centrifuging samples for 25 min at 18,500 x g and 4°C [25-27, 35]. We further purified the seminal fluid by removing the supernatant from the sperm pellet and centrifuging for 10 min at 18,500 x g and 4°C. Next we removed the seminal fluid and froze it at -80°C.

iTRAQ analysis for protein quantitation of SF from *N. apis*-infected drones.

We quantified seminal fluid proteins for each of the 8 seminal fluid samples (4x *N. apis*-infected and 4x control) using a Bradford assay (Thermo Scientific, #B14, USA) according to manufacturer's instructions. For each of the samples, we precipitated 50 µg of protein, by adding

six volumes of cold acetone and then stored them at -20°C overnight. Next, we centrifuged the precipitated proteins at 20,000 x g for 20 min, washed the pellet in cold acetone twice, removed the supernatant and suspended the resulting pellet in dissolution buffer (iTRAQ® Reagent - Multiplex Buffer Kit). Next, we denatured the proteins and blocked cysteines according to the manufacturer's instructions. Each sample was digested with 5 µg of trypsin (Promega, Australia) at 37° C overnight and labelled with iTRAQ reagent (according to the manufacturer's instructions, iTRAQ® Reagent - 8PLEX Multiplex Kit) with 4 replicates in the following experiment: control- 113, 115, 117 and 119; *N. apis*-infected- 114, 116, 118 and 121. The labelled samples were pooled prior to further analysis. To remove excess labelling reactants and to reduce interference of salts during LC-MS/MS analysis, as well as to reduce their complexity, pooled samples were separated into 35x 1 min fractions over a 35 min gradient 0-60 % buffer B (10 mM KHPO4 in 1 M KCl and 25 % [v/v] ACN, pH 3) using a SCX column (4.6 mm, 10 cm, 300 A, PolyLC, Columbia, USA). The fractions were dried in vacuo and desalted using C18 cartridges (Nest Group, USA).

Mass spectrometry and database searching

We resuspended the 20 active fractions in 5% ACN and 0.1% formic acid (FA) water and analysed them in an Agilent 6550 Q-TOF mass spectrometer with an HPLC-Chip Cube source. A large capacity chip was used for online separation (160 nL enrichment column (Zorbax 300SB-C18 5 µm, USA) driven by an Agilent Technologies 1200 series HPLC system. We loaded peptides onto the trapping column at 3 µL/min in 2 % (v/v) ACN and 0.1 % (v/v) FA and eluted them over the analytical column with a flow-rate of 300 nL/min during a 60min gradient (2-45 % ACN, 0.1% FA) directly into the mass spectrometer. The Q-ToF was run in positive ion mode and MS scans run

over a range of m/z 300-2000 and at a scan rate of 4 spectra/sec. Precursor ions were selected for auto MS/MS at an absolute threshold of 500 and a relative threshold of 0.01, with max 3 precursors per cycle, and active exclusion set at 2 spectra and released after 1 min. Precursor charge-state selection and preference was set to 2+ and 3+ and precursors were selected by charge then abundance. Using MassHunter Qualitative Analysis we extracted and exported resulting MS/MS spectra as mgf files. We searched the resulting files against the official gene set for honey bee curated by the BeeBase consortium (amel_OGS_3.2, release 60, date downloaded: 28th October 2013, with 16031 protein sequences) available here: http://hymenopteragenome.org/beebase; including number of common contaminants downloaded from GPM FTP site (http://www.thegpm.org/crap) using Mascot version 2.5.1 (Matrix Science). We used the following Mascot settings: MS error tolerance ± 100 ppm, MS/MS error tolerance ±0.5 Da, maximum missed cleavages tolerated as 1, peptide charge as 2+, 3+ and 4+ and finally, the instrument selected as ESI-Q-ToF. For quantification we used the quantification editor for iTRAQ reporter ions as specified in Mascot: including fixed modifications methylthio (C), iTRAQ8plex (N-term), iTRAQ8plex (K) and variable modifications oxidation (M) and iTRAQ8plex (Q). In order to increase the number of identifications, the peptide list generated from Mascot was exported and then used as an exclusion list (based on the peptide (*m/*z) and charge (z)) for a repeat the Q-ToF run of the same fraction [36]. Multiple SCX fractions and exclusion runs with mzdataCombinator v1.0.4 (The West Australian Centre of Excellence in Computational Systems Biology). For calculation of the FDR, we used the decoy function within Mascot. Initially we selected proteins with a protein score >38 for identification. For all proteins identified with only 1 peptide the MS/MS spectrum was reviewed and 49 proteins with low peptide ion scores and poor b-, y-ion series were removed from further analyses.

For the Mascot Quantitative analysis of the iTRAQ tags the following settings were used: average protein ratios, median ratio normalisation with no outlier removal, minimum precursor charge +2, and minimum number of peptides set to 2. For each ratio, the mean of all reported values for peptide matches was converted to one and normalisation was performed at the peptide level. We quantified protein abundances; comparing the 4 samples of seminal fluid from *N. apis*-infected males to the 4 controls collected from sugar-fed males. Because Mascot is not able to compare protein abundances of peptide distributions that are not normally distributed, we exported the peptide ratios for these proteins to SPSS (v.23) and analysed the data with *One-Sample Wilcoxon Signed Rank Tests*. Because iTRAQ is known to underestimate the difference in abundance between proteins due to "Ratio compression", isotopic contamination and background interference[37-39], we included all proteins that were identified as significantly different between treatments for further analysis. The results exported from Mascot can be seen in the Supplementary Table S2.

Protein annotation, functional grouping, and localization

BeeBase accessions were annotated using a BLAST search against the NCBI Refseq protein database (accessed October 2013). We based the functional groups for each protein on annotations of the best BLASTp analysis of the matching amino acid sequences against *Drosophila melanogaster* and on searches through function descriptions in UniProtKB and KEGG. Next, we searched in PubMed with the keywords: insect and immune, and if an immune response was described in insects, we accepted this classification.

To predict the subcellular location of the identified proteins, we used TargetP 1.1. The location assignment predicts the presence and location of signal peptide cleavage sites in amino acid sequences[40], for classical signals peptide identification (a neural network based scoring function where >0.5 indicates possible secretion). Furthermore, we compared all identified proteins with previous publications on insect seminal fluid, including honey bee *A. mellifera* [25, 41], the fall field cricket *Gryllus pennsylvanicus* [42, 43], the Pacific field cricket *Teleorgyllus oceanicus* [44], the fruit fly *D. melanogaster* [45-47], the yellow fever mosquito *Aedes aegypti* [48], the bedbug *Cimex lectularius* [49] as well as several butterflies in the genus *Heliconius* [50], and by standard BLASTP analysis. Proteins, previously described in sperm or seminal fluid, were classified accordingly, regardless of their TargetP score. Finally, we classified proteins as secreted if their localisation was described as extracellular within GO, KEGG or UniProtKB. After functional annotation, we compared the number of significantly changed proteins versus unchanged proteins in each category using a *Fisher's Exact* test (http://www.langsrud.com/stat/fisher.htm). *Fisher's Exact* tests were also used to compare the number of protein increased versus decreased in abundance.

Quantifying the antimicrobial activity of seminal fluid against *N. apis*

We incubated samples of 5 x 10^5 *N. apis* spores for 5 min in 5 µl of pure seminal fluid from *N. apis*-infected or uninfected drones, as well as 5x, 10x and 20x dilutions of seminal fluid of both treatments in Hayes, as well as pure Hayes as a negative control. Spores were incubated with seminal fluid for 5 min, which was previously shown to be sufficient to quantify maximal spore killing rate [26]. The viability of *N. apis* spores was quantified using flow cytometry as described in Peng *et al*. [32]. In brief, spores were stained with 5 µM SYTO (16) green and 0.02 µM SYTOX red

fluorescent nucleic acid dyes and then live and dead spores were quantitated in a BD FACSCalibur flow cytometer. Emission from SYTO Green was collected using a 530/30 BP filter, and SYTOX red emission with a 670/30 BP filter. We analysed samples on a low flow-rate setting (12 ± 3 µl/min), counted 10,000 spores and identified live and dead spores using pulse height for SYTO green SYTOX red, without compensation. Data were analysed using FlowJo Version 7.6.5 for Windows (TreeStar, USA).

Quantification of the effect of seminal fluid on sperm viability

Sperm viability of infected and non-infected males was compared using a recently developed flow cytometry method [51]. To do this, we raised 500 drones in four additional honey bee colonies headed by non-related queens in spring 2014, and fed hatching individuals with 10,000 *N. apis* spores or sugar water as a control. We kept males afterwards in wooden cages in groups of up to 140 males, separated into treatment and control and returned them back into their maternal colonies, as described previously [8, 32]. Drone survival per cage was 70-90%, comparable to earlier findings and seemed unaffected by treatment. We retrieved the males 12 days later and sampled ejaculates, using a pipette tip moistened with 1 µL of semen diluent (188.3 mM NaCl, 5.6 mM glucose, 574.1 nM arginine, 684.0 nM lysine, 50 mM tris (hydroxymethyl) aminomethane, pH 8.7), which we further diluted with 1 ml of semen diluent. To avoid mucus clogging up the capillary of the flow cytometer, we filtered a subsample of 200 µl semen sample collected as described above through a 50 µm diameter nylon mesh. After a further dilution with 800 µl of semen diluent, we used 400 µl of sperm sample and dyed the sperm with SYBR 14 dye (Invitrogen) and Propidium Iodide, PI (Invitrogen), counting a minimum of 3000 sperm in a BD FACSCanto II digital flow cytometer. We recorded

SYBR 14 fluorescent emission in the range 515-545 nm, and PI fluorescent emission in the range 670-735 nm, without compensation for spectral overlap and recorded height rather than area of the voltage pulse. Sperm dyed with SYBR 14 were gated as live and those stained with PI were gated as dead, using the FlowJo software package Version 7.6.5 for Windows (TreeStar, USA); for more detail see Paynter *et al* [51]. To analyse the effects of treatment and colony on sperm viability, we first removed 3 outliers with sperm viabilities below 30% caused by the filtration, then normalised the variable calculating its arcsin-sqrt transformation and performed an ANOVA using SPSS version 23 for Macintosh.

Results

Protein abundance in seminal fluid of infected and non-infected males

To compare protein abundances between infected and non-infected males, we used iTRAQ for a quantitative proteomic analysis and identified a total of 260 seminal fluid proteins with high confidence (Supplemental Table S1 for details). We used isobaric tags after MS/MS fragmentation for quantification within Mascot. Of these, abundance was significantly higher for 46 seminal fluid proteins, whereas 65 protein abundances were significantly lower in infected compared to non-infected males. This core responsive group of 111 proteins was further analysed and grouped according to their gene ontology and described protein function in the literature to define putative mechanisms of change in seminal fluid potency (Table 1). The largest functional group consisted of 15 immune proteins, which changed significantly in abundance in infected males (Table 1). A

Fisher's Exact test revealed that immune proteins were significantly overrepresented in the whole dataset and changed significantly more often in abundance compared to immune proteins that did not (15 versus 5, $p < 0.01$). The second largest functional group in the core responsive group consisted of 14 proteins associated with defence against reactive oxygen species (ROS), 6 of which increased and 8 decreased significantly in protein abundance. Additionally, the abundance of 9 out of 10 proteins involved in glycolysis was significantly decreased in the seminal fluid of infected males (Table 1).

An extracellular localization confirmed 57 proteins out of the core responsive group to be extracellular secretions, based on TargetP predicted secretory peptides or an extracellular localisation description in UniProtKB (Table 2). When we analysed these 57 proteins in more detail, we found secretory tags for all of the 15 proteins with documented links to insect immune responses. These included well-known immune response proteins such as chitinase 5, peritrophin-1-like isoform X1, osiris 7, C1q-like venom protein, apyrase, glucose dehydrogenase 2 and glycosinolate sulphatase as well as apolipophorins-like proteins. Also in significantly higher abundance in infected males, were Niemann-Pick disease type C2 proteins (NPC2), which have been described as part of a signalling cascade activating the Immune Deficiency (IMD) pathway against pathogens in insects [52]. Additionally, we identified 6 proteases/peptidases such as aminopeptidases N and metallopeptidases, which have previously been found to be associated with immune response, activating the production of AMPs in insects [53-56].

The remaining 54 proteins of the core responsive group that did not have secretory tags, included 16 previously identified sperm proteins and 38 proteins of intracellular origin. The majority of these

proteins were found in lower abundance in the seminal fluid of infected males (Table 2), including a number of ROS response proteins. Despite their cellular origin, ROS-response proteins, in particular superoxide dismutase and thioredoxin peroxidase 4, have previously been associated with a humoral immune response in insects [57]. The list of sperm-associated proteins indicate a loss of respiratory capacity and the abundance change of proteins involved in amino acid metabolism imply a response to *N. apis* infection (Table 2).

Antimicrobial activity of seminal fluid against *N. apis* spores

When we compared the effect of seminal fluid collected from *N. apis*-infected and non-infected males on *N. apis* spore viability, we found that seminal fluid of both treatments significantly reduced the viability of *N. apis* spores, killing up to 90% of spores (Figure 1). This effect was also found in diluted samples of seminal fluid (ANOVA, F (dilution) $_{3, 21} = 3930.569$, $p < 0.01$). Overall, seminal fluid of infected males killed significantly fewer spores compared to seminal fluid of control males (ANOVA, F (treatment)$_{1, 21} = 663.421$, $p < 0.01$, Figure 1).

Effects of *N. apis* infections on sperm viability

Sperm viabilities between *N. apis*-infected males (n=34) and males fed with sugar syrup (n=36) were not significantly different (ANOVA, F = 1.134, $p = 0.291$ *n.s.*). This indicates that the changes in protein abundance in seminal fluid of infected males were unlikely to be driven by differences in the proportion of dead or ruptured sperm cells present in the ejaculates. This is consistent with previous work showing that *N. apis* infections in young males have no major pathological effect, and that fertility of infected males is only affected in older males (24 days) [15]. Similarly, the survival

of males did not differ between infected and control males, which is in line with our previous work that only found increases in mortality of infected males that were 19 days or older [15].

Discussion

Infections of the fungal pathogen *N. apis* trigger substantial alterations in the seminal fluid proteome of honey bees as indicated by significant abundance changes in 43% of proteins we were able to quantify. We found that the largest group of proteins with changed abundances consisted of 31 proteins with well-established links to insect immunity (Table 3). As we point out in the paragraphs below, this immune response in the seminal fluid of infected honey bees affected key proteins of the insect immune system.

Insects such as honey bees have well developed innate immune systems, which consist of cellular responses involving fat body cells or haemocytes as well as humoral responses. The latter is made up of immune proteins and peptides that form key biochemical pathways of insect defences (Figure 2), such as the Toll Pathway [57], Immune Deficiency (IMD) [57, 59], c-Jun N-terminal kinases (JNK) signalling and Janus kinase/signal transducers and activators of transcription (JAK/STAT) [60]. The Toll pathway is predominantly activated by Gram-positive bacteria, fungi, and yeast, whereas the IMD pathway is triggered by Gram-negative bacteria [61]. Stress and injury induce the JAK/STAT pathway via cytokines that bind to the receptor Domeless and stimulate cellular responses such as cell cycle regulation and apoptosis (Bordenstein Lab, NSF DEB-1046149). Several of the immune proteins we identified during our experiments are associated with humoral immune response

pathways, such as the Toll pathway, JAK/STAT pathway, proPO cascade, and ROS-induced humoral immune responses that also activate cellular responses (Figure 2). The fact that we found abundance changes in immune proteins that belong to different pathways of the innate insect immunity implies that the immune system present in the seminal fluid is remarkably complex and overlaps substantially with the innate immune system found in other somatic body parts of insects (Table 3).

Of the 15 secreted immune response proteins detected in our experiment, 12 increased in abundance in response to an infection. They are well-characterized proteins of humoral immunity, including 2 chitinases and peritrophin-1 like protein, which binds chitin. Insect chitinases are part of the Toll pathway [86, 97] and are able to break down the chitin cell walls of parasitic fungi [24, 62, 98]. Transcript levels of Group IV chitinases have been shown to increase in response to *N. ceranae* infections in honey bee workers [93]. Peritrophin-1 like has also been found in the gut of insects where it has been proposed as a defence molecule against microorganisms [66]. Osiris 7 was the only protein from the Toll pathway that we found in lower abundance in seminal fluid of infected drones. This protein is a peptidoglycan recognition protein and key for the initial recognition reaction of pathogens, resulting in the activation of the Toll pathway [57, 99]. One reason for the observed lower abundance could be that the pathogen is able to suppress host recognition in order to establish an infection [70], which would be consistent with previous research[74, 88, 95, 99]. Fungal pathogens have also been described to activate the Prophenol-oxidase (proPO) cascade, and we indeed identified a number of proteins involved in this immune response leading to melanisation and subsequent encapsulation of the invading organism. Apolipophorin-like protein, matrix metalloproteinase 25 and a renin receptor showed reduced abundance in the seminal fluid of infected males. Apolipophorins can

function as immune response proteins [75, 79, 80] and are able to initiate the production of antimicrobial peptides [79]. To do this, ApoLp-III binds to microbial cell wall components including fungal α-1,3-glucan, and activates a multicellular encapsulation reaction via the insect's proPO cascade [80]. Apolipoproteins as well as matrix metalloproteinases can also activate antimicrobial peptides, such as defensin [79], the latter being a key element of the insects' innate immune system against bacteria and fungi. Although we were unable to identify smaller immune peptides such as defensin, the presence of proteins known to activate and modify AMPs indicates the release of these compounds into the seminal fluid. AMPs are produced in the fat body from where they are released into the hemolymph in response to infections [100-103]. AMP gene expression is activated by two distinct signalling pathways, the Toll pathway and the IMD pathway (Figure 2).

Humoral immune responses are also controlled by proteins involved in reaction to oxidative stress (ROS) defence [57], because ROS are produced in the midgut cells and act at sites of infections to directly kill pathogens or as signals of cellular stress to activate humoral immune responses such as encapsulation and melanisation [86, 89, 90, 104, 105]. We detected abundance changes in a number of proteins associated with a reaction to oxidative stress (Table 3). Many of these proteins have previously been reported as molecular components of a humoral immune response in insects. Furthermore, they have been detected to be secreted into other fluids such as venom [86, 89, 90]. Production of AMPs and ROS, as a response to bacterial and fungal infections in the gut of insects [92], have been reported for may insects, including the cockroach *Blaberus discoidalis* [106], the silkworm *Bombyx mori* [107], the greater wax moth *Galleria mellonella* [108] and the tiger moth *Parasemia plantaginis* [109]. In the mosquito, high levels of ROS are required to evoke a reaction to the malaria parasite *Plasmodium* [89].

Activation of the Toll pathway leads to the transcription and release of AMPs and chitin binding enzymes. A release into the haemolymph has previously been shown for activation by fungal infections [90]. Here we identified a number of these active proteins in the seminal fluid of male honey bees, indicating that the seminal fluid is also protected by these active components. An additional 4 proteolytic proteins were found in higher abundance in infected males, 2 of these have well documented immune activities (Table 3). Antitrypsin and aminopeptidase N are both proteins involved in the innate immune pathway in honey bees and are involved in encapsulation and melanisation [55, 110, 111].

As pointed out above, our findings imply that males respond to *N. apis* infections with a complex immune response that is present, at least in part, in their seminal fluid (Table 3). The activation of key immune response proteins, following a *N. apis* infection, has previously been found in honey bee workers [93], but we provide a detailed insight into the immune proteome response of males in their seminal fluid. Our findings imply an adaptive immune response of males to minimize disease transmission during mating by a targeted increase of antifungal immunity, which could trade off with a reduction in immune investment into non-fungal defences. This may be further supported by the reduction on proteins of the proPO cascade (Table 3). Alternatively, *N. apis* may be able to suppress parts of the honey bee immune system as part of its propagation in the host and resulting in the up-regulation of alternative immune pathways in males to combat the parasite. Immune suppression by parasites is indeed a widespread phenomenon [112-114] and has also been reported for *Nosema ceranae* [93]. Our experiment quantifying the antifungal activity of seminal fluid of infected and non-infected males provided support of the latter case because we found that infected males were still able to kill *N. apis* spores with remarkably high efficiency, although their spore killing capacity was significantly lower in diluted seminal fluid compared to seminal fluid of non-infected

males. These findings are interesting for two reasons: Firstly, we found no significant difference in spore killing between pure seminal fluid samples of infected and non-infected males (Figure 1). This implies that males maintain maximal antimicrobial activity against *N. apis* irrespectively of their infection status. Secondly, changes in protein abundance of immune system proteins (Table 2) imply that the infected male undergoes a dynamic shift to maintain optimal pathogen defence as well as fecundity, in spite of being infected and possibly manipulated by the parasite. Honey bee queens are known for their exceptionally high polyandrous behaviour [6], and polyandry favours the evolution of sexually transmittable diseases [10]. Our data therefore implies that males maintain high antimicrobial activity in their seminal fluid due to an elevated risk that their ejaculates or the queen they copulate with will come into contact with contaminated semen. This confirms earlier work, where we showed that seminal fluid is of central importance to keep sperm alive and that environmental stressors, including parasites, mainly target male survival but not male fertility [15]. We therefore conclude that some of the proteomic changes we observed in the secreted proteins within the seminal fluid are an adaptive response to keep sperm alive, if cellular contaminations pose a potential risk for sperm cells.

Conclusion

Our data show the immune system of honey bee males is substantially more complex and plastic than acknowledged so far. Our findings also offer a number of opportunities for future research to further understand the complex interplay between the honey bee immune system and the molecular machinery determining the virulence of *N. apis*. Firstly, we can now design more targeted

experiments to test whether the proteins of the core responsive group are involved in proximate mechanisms of host-parasite interactions. Secondly, we can start disentangling which molecular effects are caused by parasite virulence and which by host resistance. Finally, our results confirm our previously published work [26] in which we found that honey bee males use at least two distinctly different molecular pathways in their seminal fluid to neutralise *N. apis* spores and provide putative identities of the agents for both pathways.

FIGURES

Figure 1: The effect of seminal fluid of infected and non-infected males on *N. apis* spore survival compared to a Hayes control treatment without any antimicrobial activity. Although, males of both treatments significantly reduced spore viability, seminal fluid of infected males was less potent to kill spores compared to seminal fluid of non-infected males (ANOVA, F (treatment)$_{1, 21} = 663.421$, $p < 0.01$). This effect was also found in diluted samples of seminal fluid (ANOVA, F (dilution) $_{3, 21} = 3930.569$, $p < 0.001$).

Figure 2: A schematic diagram of the insect immune system. Different pathogens and stresses are recognised through lipopolysaccharides, peptidoglycans, β-1,3-glycans. Infection activates a number of different immune pathways inside the cell. The Toll, Immune Deficiency (IMD), c-Jun N-terminal kinases (JNK) signaling, Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathways and humoral response are activated by proteins such as Osiris 7, the

Peptidoglycan recognising protein (PGRP). Cellular responses include phagocytosis, cell cycle response and cytoplasmic antimicrobial peptides (cAMPs). Extracellular responses, such as AMPs, chitinases, melanisation and encapsulation attack the pathogen in the haemolymph, seminal fluid and venom. Reactive oxygen species (ROS) activate the humoral immune response at the site of injury and cause melanisation and the release of AMPs. Proteins with * were significantly changed in our dataset.

ASSOCIATED CONTENT

Supplementary Table S1: Identification and quantitation of proteins in seminal fluid of *N. apis*-infected and un-infected males. For every protein the Mascot database search results, functional Category, quantitative proteins summary, cellular localisation, Citations and TargetP localisation are shown.

Supplementary Table S2: Peptide summary and Protein quantification summary exported from Mascot database search.

a. For the peptide summary, for every protein, we show all peptides that were identified and quantitated using the reporter ion intensities. For quantification we show the reporter ions for every peptide (Control: 113, 115, 117, 119) / (*N. apis* infected: 114,116,118,121).

b. For the protein quantification, we show the average reporter ion ratios for each protein calculating *p*-value in Mascot for normally distributed and in SPSS for non-normal.

AUTHOR INFORMATION

***Corresponding Author**

Julia Grassl

Phone +61 8 6488 4411

Fax +61 8 6488 1029

Email: julia.grassl@uwa.edu.au

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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REFERENCES

(1) Schmid-Hempel, P., *Evolutionary parasitology - the integrated study of infections, immunology, ecology, and genetics*. 2011, Oxford: Oxford Univerity Press.

(2) Gerloff, C.U., B.K. Ottmer, and P. Schmid-Hempel, Effects of inbreeding on immune response and body size in a social insect, *Bombus terrestris. Funct Ecol*, **2003**, *17*, 582-589.

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

(4) Vainio, L., H. Hakkarainen, M.J. Rantala, and J. Sorvari, Individual variation in immune function in the ant *Formica exsecta*; effects of the nest, body size and sex. *Evol Ecol*, **2004**, *18*, 75-84.

(5) O'Donnell, S. and S.N. Beshers, The role of male disease susceptibility in the evolution of haplodiploid insect societies. *Proc Biol Sci*, **2004**, *271*, 979-983.

(6) Baer, B., *Sexual Selection in Social Insects*, in *The Evolution of Insect Mating Systems*, D. Shuker and L.W. Simmons, Editors. 2014, Oxford University Press: Oxford. p. 261-274.

(7) Baer, B., The copulation biology of ants (Hymenoptera: *Formicidae*). *Myrmecol. News*, **2011**, *14*, 55-68.

(8) Sturup, M., B. Baer-Imhoof, D.R. Nash, J.J. Boomsma, and B. Baer, When every sperm counts: factors affecting male fertility in the honeybee *Apis mellifera. Behav Ecol*, **2013**, *24*, 1192-1198.

(9) Sturup, M., S.P.A. den Boer, D.R. Nash, J.J. Boomsma, and B. Baer, Variation in male body size and reproductive allocation in the leafcutter ant *Atta colombica*: estimating variance components and possible trade-offs. *Insect Soc*, **2011**, *58*, 47-55.

(10) Knell, R.J. and K.M. Webberley, Sexually transmitted diseases of insects: distribution, evolution, ecology and host behaviour. *Biol Rev Camb Philos Soc*, **2004**, *79*, 557-581.

(11) de Miranda, J.R. and I. Fries, Venereal and vertical transmission of deformed wing virus in honeybees (*Apis mellifera* L.). *J Invertebr Pathol*, **2008**, *98*, 184-189.

(12) Yue, C., M. Schroder, K. Bienefeld, and E. Genersch, Detection of viral sequences in semen of honeybees (*Apis mellifera*): evidence for vertical transmission of viruses through drones. *J Invertebr Pathol*, **2006**, *92*, 105-108.

(13) Yue, C., M. Schroder, S. Gisder, and E. Genersch, Vertical-transmission routes for deformed wing virus of honeybees (*Apis mellifera). J Gen Virol*, **2007**, *88*, 2329-2336.

(14) Baer, B. and A.H. Millar, Proteomics in evolutionary ecology. *J Proteomics*, **2016**, *135*, 4-11.

(15) Peng, Y., B. Baer-Imhoof, A.H. Millar, and B. Baer, Consequences of *Nosema apis* infection for male honey bees and their fertility. *Sci Rep*, **2015**, *5*, 10565.

(16) Bailey, L., *Nosema apis* in drone honeybees. *J. Apic. Res. (1962-2005)*, **1972**, *11*, 171-174.

(17) Zander, E., Tierische Parasiten als Krankheitserreger in der Biene. *Leipziger Bienenzeitung*, **1909**, *24*, 147-150.

(18) De Graaf, D.C., H. Raes, G. Sabbe, P.H. De Rycke, and F.J. Jacobs, Early Development of *Nosema apis* (Microspora: *Nosematidae*) in the Midgut Epithelium of the Honeybee (*Apis mellifera*). *J Invertebr Pathol*, **1994**, *63*, 74-81.

(19) Wittner, M. and L.M. Weiss, *The microsporidia and microsporidiosis*. 1999, Washington: ASM press.

(20) Liu, T.P., The release of *Nosema apis* spores from the epithelium of the honeybee gut. *J. Apic. Res. (1962-2005)*, **1990**, *29*, 221-229.
(21) Graham, M.J., *The hive and the honey bee*. Seventh edition ed. 2005, Hamilton, Illinois: Dadant & Sons.
(22) Roberts, K.E., S.E. Evison, B. Baer, and W.O. Hughes, The cost of promiscuity: sexual transmission of Nosema microsporidian parasites in polyandrous honey bees. *Scientific Reports*, **2015**, *5*, 10982.
(23) Kramer, K.J. and S. Muthukrishnan, Insect chitinases: Molecular biology and potential use as biopesticides. *Insect Biochem Molec*, **1997**, *27*, 887-900.
(24) Vega, K. and M. Kalkum, Chitin, chitinase responses, and invasive fungal infections. *Int J Microbiol*, **2012**, *2012*, 920459.
(25) Baer, B., J.L. Heazlewood, N.L. Taylor, H. Eubel, and A.H. Millar, The seminal fluid proteome of the honeybee *Apis mellifera. PROTEOMICS*, **2009**, *9*, 2085-2097.
(26) Peng, Y., J. Grassl, A.H. Millar, and B. Baer, Seminal fluid of honeybees contains multiple mechanisms to combat infections of the sexually transmitted pathogen *Nosema apis. Proc Biol Sci*, **2016**, *283*.
(27) Baer, B., R. Zareie, E. Paynter, V. Poland, and A.H. Millar, Seminal fluid proteins differ in abundance between genetic lineages of honeybees. *J Proteomics*, **2012**, *75*, 5646-5653.
(28) John Roberts , RIRDC Publication No 15/095, *Upgrading knowledge on pathogens (particularly viruses) of Australian honey bees*, R.I.R.a.D. Corporation, Editor 2015: Australia. p. 22.
(29) Williams, G.R., D. Shutler, C.M. Little, K.L. Burgher-MacLellan, and R.E.L. Rogers, The microsporidian *Nosema ceranae*, the antibiotic Fumagilin-B (R), and western honey bee (*Apis mellifera*) colony strength. *Apidologie*, **2011**, *42*, 15-22.
(30) Williams, G.R., D. Shutler, K.L. Burgher-MacLellan, and R.E. Rogers, Infra-population and -community dynamics of the parasites *Nosema apis* and *Nosema ceranae*, and consequences for honey bee (*Apis mellifera*) hosts. *PLoS One*, **2014**, *9*, e99465.
(31) Milbrath, M.O., T. van Tran, W.F. Huang, L.F. Solter, D.R. Tarpy, F. Lawrence, and Z.Y. Huang, Comparative virulence and competition between *Nosema apis* and *Nosema ceranae* in honey bees (*Apis mellifera*). *J Invertebr Pathol*, **2015**, *125*, 9-15.
(32) Peng, Y., T.F. Lee-Pullen, K. Heel, A.H. Millar, and B. Baer, Quantifying spore viability of the honey bee pathogen *Nosema apis* using flow cytometry. *Cytometry A*, **2014**, *85*, 454-462.
(33) Cantwell, G.E., Standard methods for counting nosema spores. *Am Bee J*, **1970**, *110*, 222-223.
(34) Schley, P., *Einführung in die Technik der instrumentellen Besamung von Bienenköniginnen*. 2 ed. 1987, Giessen: Köhler Offset KG. 103.
(35) King, M., H. Eubel, A.H. Millar, and B. Baer, Proteins within the seminal fluid are crucial to keep sperm viable in the honeybee *Apis mellifera. J Insect Physiol*, **2011**, *57*, 409-414.
(36) Eubel, H., E.H. Meyer, N.L. Taylor, J.D. Bussell, N. O'Toole, J.L. Heazlewood, I. Castleden, I.D. Small, S.M. Smith, and A.H. Millar, Novel proteins, putative membrane transporters, and an integrated metabolic network are revealed by quantitative proteomic analysis of Arabidopsis cell culture peroxisomes. *Plant Physiol*, **2008**, *148*, 1809-1829.
(37) Ow, S.Y., M. Salim, J. Noirel, C. Evans, I. Rehman, and P.C. Wright, iTRAQ underestimation in simple and complex mixtures: "the good, the bad and the ugly". *J Proteome Res*, **2009**, *8*, 5347-5355.

(38) Karp, N.A., W. Huber, P.G. Sadowski, P.D. Charles, S.V. Hester, and K.S. Lilley, Addressing accuracy and precision issues in iTRAQ quantitation. *Mol Cell Proteomics*, **2010**, *9*, 1885-1897.
(39) Trinh, H.V., J. Grossmann, P. Gehrig, B. Roschitzki, R. Schlapbach, U.F. Greber, and S. Hemmi, iTRAQ-Based and Label-Free Proteomics Approaches for Studies of Human Adenovirus Infections. *Int J Proteomics*, **2013**, *2013*, 581862.
(40) Emanuelsson, O., H. Nielsen, S. Brunak, and G. von Heijne, Predicting subcellular localization of proteins based on their N-terminal amino acid sequence. *J Mol Biol*, **2000**, *300*, 1005-1016.
(41) Poland, V., H. Eubel, M. King, C. Solheim, A. Harvey Millar, and B. Baer, Stored sperm differs from ejaculated sperm by proteome alterations associated with energy metabolism in the honeybee *Apis mellifera. Mol Ecol*, **2011**, *20*, 2643-2654.
(42) Andres, J.A., L.S. Maroja, S.M. Bogdanowicz, W.J. Swanson, and R.G. Harrison, Molecular evolution of seminal proteins in field crickets. *Mol Biol Evol*, **2006**, *23*, 1574-1584.
(43) Braswell, W.E., J.A. Andres, L.S. Maroja, R.G. Harrison, D.J. Howard, and W.J. Swanson, Identification and comparative analysis of accessory gland proteins in *Orthoptera. Genome Biol*, **2006**, *49*, 1069-1080.
(44) Simmons, L.W., Y.F. Tan, and A.H. Millar, Sperm and seminal fluid proteomes of the field cricket *Teleogryllus oceanicus*: identification of novel proteins transferred to females at mating. *Insect Mol Biol*, **2013**, *22*, 115-130.
(45) Dorus, S., S.A. Busby, U. Gerike, J. Shabanowitz, D.F. Hunt, and T.L. Karr, Genomic and functional evolution of the *Drosophila melanogaster* sperm proteome. *Nat Genet*, **2006**, *38*, 1440-1445.
(46) Findlay, G.D., X.H. Yi, M.J. MacCoss, and W.J. Swanson, Proteomics reveals novel *Drosophila* seminal fluid proteins transferred at mating. *PLoS Biology*, **2008**, *6*, 1417-1426.
(47) Wasbrough, E.R., S. Dorus, S. Hester, J. Howard-Murkin, K. Lilley, E. Wilkin, A. Polpitiya, K. Petritis, and T.L. Karr, The *Drosophila melanogaster* sperm proteome-II (DmSP-II). *J Proteomics*, **2010**, *73*, 2171-2185.
(48) Sirot, L.K., M.C. Hardstone, M.E. Helinski, J.M. Ribeiro, M. Kimura, P. Deewatthanawong, M.F. Wolfner, and L.C. Harrington, Towards a semen proteome of the dengue vector mosquito: protein identification and potential functions. *PLoS Negl Trop Dis*, **2011**, *5*, e989.
(49) Otti, O., A.P. McTighe, and K. Reinhardt, In vitro antimicrobial sperm protection by an ejaculate-like substance. *Funct Ecol*, **2013**, *27*, 219-226.
(50) Walters, J.R. and R.G. Harrison, Combined EST and proteomic analysis identifies rapidly evolving seminal fluid proteins in *Heliconius* butterflies. *Mol Biol Evol*, **2010**, *27*, 2000-2013.
(51) Paynter, E., B. Baer-Imhoof, M. Linden, T. Lee-Pullen, K. Heel, P. Rigby, and B. Baer, Flow cytometry as a rapid and reliable method to quantify sperm viability in the honeybee *Apis mellifera. Cytometry A*, **2014**, *85*, 463-472.
(52) Shi, X.Z., X. Zhong, and X.Q. Yu, *Drosophila melanogaster* NPC2 proteins bind bacterial cell wall components and may function in immune signal pathways. *Insect Biochem Mol Biol*, **2012**, *42*, 545-556.

(53) Wilson, C.L., A.P. Schmidt, E. Pirila, E.V. Valore, N. Ferri, T. Sorsa, T. Ganz, and W.C. Parks, Differential Processing of {alpha}- and {beta}-Defensin Precursors by Matrix Metalloproteinase-7 (MMP-7). *J Biol Chem*, **2009**, *284*, 8301-8311.
(54) Reinhold, D., A. Biton, A. Goihl, S. Pieper, U. Lendeckel, J. Faust, K. Neubert, U. Bank, M. Tager, S. Ansorge, and S. Brocke, Dual inhibition of dipeptidyl peptidase IV and aminopeptidase N suppresses inflammatory immune responses. *Ann N Y Acad Sci*, **2007**, *1110*, 402-409.
(55) Candas, M., O. Loseva, B. Oppert, P. Kosaraju, and L.A. Bulla, Jr., Insect resistance to *Bacillus thuringiensis:* alterations in the indianmeal moth larval gut proteome. *Mol Cell Proteomics*, **2003**, *2*, 19-28.
(56) Galindo, R.C., E. Doncel-Perez, Z. Zivkovic, V. Naranjo, C. Gortazar, A.J. Mangold, M.P. Martin-Hernando, K.M. Kocan, and J. de la Fuente, Tick subolesin is an ortholog of the akirins described in insects and vertebrates. *Dev Comp Immunol*, **2009**, *33*, 612-617.
(57) Christophides, G.K., E. Zdobnov, C. Barillas-Mury, E. Birney, S. Blandin, C. Blass, P.T. Brey, F.H. Collins, A. Danielli, G. Dimopoulos, C. Hetru, N.T. Hoa, J.A. Hoffmann, S.M. Kanzok, I. Letunic, E.A. Levashina, T.G. Loukeris, G. Lycett, S. Meister, K. Michel, L.F. Moita, H.M. Muller, M.A. Osta, S.M. Paskewitz, J.M. Reichhart, A. Rzhetsky, L. Troxler, K.D. Vernick, D. Vlachou, J. Volz, C. von Mering, J. Xu, L. Zheng, P. Bork, and F.C. Kafatos, Immunity-related genes and gene families in *Anopheles gambiae. Science*, **2002**, *298*, 159-165.
(58) Zareie, R., H. Eubel, A.H. Millar, and B. Baer, Long-term survival of high quality sperm: insights into the sperm proteome of the honeybee *Apis mellifera. J Proteome Res*, **2013**, *12*, 5180-5188.
(59) The Nasonia Genome Working, G., Functional and Evolutionary Insights from the Genomes of Three Parasitoid Nasonia Species. *Science (New York, N.Y.)*, **2010**, *327*, 343-348.
(60) Gerardo, N.M., B. Altincicek, C. Anselme, H. Atamian, S.M. Barribeau, M. de Vos, E.J. Duncan, J.D. Evans, T. Gabaldon, M. Ghanim, A. Heddi, I. Kaloshian, A. Latorre, A. Moya, A. Nakabachi, B.J. Parker, V. Perez-Brocal, M. Pignatelli, Y. Rahbe, J.S. Ramsey, C.J. Spragg, J. Tamames, D. Tamarit, C. Tamborindeguy, C. Vincent-Monegat, and A. Vilcinskas, Immunity and other defenses in pea aphids, *Acyrthosiphon pisum. Genome Biol*, **2010**, *11*, R21.
(61) Buchon, N., N. Silverman, and S. Cherry, Immunity in *Drosophila melanogaster*-from microbial recognition to whole-organism physiology. *Nat Rev Immunol*, **2014**, *14*, 796-810.
(62) Shi, L. and S.M. Paskewitz, Identification and molecular characterization of two immune-responsive chitinase-like proteins from *Anopheles gambiae. Insect Mol Biol*, **2004**, *13*, 387-398.
(63) Aufauvre, J., B. Misme-Aucouturier, B. Vigues, C. Texier, F. Delbac, and N. Blot, Transcriptome analyses of the honeybee response to *Nosema ceranae* and insecticides. *PLoS One*, **2014**, *9*, e91686.
(64) De Gregorio, E., P.T. Spellman, G.M. Rubin, and B. Lemaitre, Genome-wide analysis of the Drosophila immune response by using oligonucleotide microarrays. *Proc Natl Acad Sci U S A*, **2001**, *98*, 12590-12595.
(65) Yan, J., Q. Cheng, S. Narashimhan, C.B. Li, and S. Aksoy, Cloning and functional expression of a fat body-specific chitinase cDNA from the tsetse fly, *Glossina morsitans morsitans. Insect Biochem Mol Biol*, **2002**, *32*, 979-989.

(66) Du, X.J., J.X. Wang, N. Liu, X.F. Zhao, F.H. Li, and J.H. Xiang, Identification and molecular characterization of a peritrophin-like protein from fleshy prawn (*Fenneropenaeus chinensis*). *Mol Immunol*, **2006**, *43*, 1633-1644.
(67) An, C., E.J. Ragan, and M.R. Kanost, Serpin-1 splicing isoform J inhibits the proSpatzle-activating proteinase HP8 to regulate expression of antimicrobial hemolymph proteins in *Manduca sexta*. *Dev Comp Immunol*, **2011**, *35*, 135-141.
(68) Levashina, E.A., E. Langley, C. Green, D. Gubb, M. Ashburner, J.A. Hoffmann, and J.-M. Reichhart, Constitutive Activation of Toll-Mediated Antifungal Defense in Serpin-Deficient Drosophila. *Science*, **1999**, *285*, 1917-1919.
(69) Kramerova, I.A., A.A. Kramerov, and J.H. Fessler, Alternative splicing of papilin and the diversity of Drosophila extracellular matrix during embryonic morphogenesis. *Dev Dyn*, **2003**, *226*, 634-642.
(70) Nation, J.L., *Immunity*. 3rd ed. Insect Physiology and Biochemistry, ed. J.L. Nation. 2015: CRC Press. 690 pages.
(71) Bordenstein_Lab. *Insect Immunity Database*. 2011 [cited 2016 May 2016]; NSF DEB-1046149:[
(72) Seaman, J.A., H. Alout, J.I. Meyers, M.D. Stenglein, R.K. Dabire, S. Lozano-Fuentes, T.A. Burton, W.S. Kuklinski, W.C.t. Black, and B.D. Foy, Age and prior blood feeding of *Anopheles gambiae* influences their susceptibility and gene expression patterns to ivermectin-containing blood meals. *BMC Genomics*, **2015**, *16*, 797.
(73) Dijkers, P.F. and P.H. O'Farrell, Drosophila calcineurin promotes induction of innate immune responses. *Curr Biol*, **2007**, *17*, 2087-2093.
(74) Reumer, A., A. Bogaerts, T. Van Loy, S.J. Husson, L. Temmerman, C. Choi, E. Clynen, B. Hassan, and L. Schoofs, Unraveling the protective effect of a Drosophila phosphatidylethanolamine-binding protein upon bacterial infection by means of proteomics. *Dev Comp Immunol*, **2009**, *33*, 1186-1195.
(75) Chung, K.T. and D.D. Ourth, Purification and characterization of apolipophorin III from immune hemolymph of *Heliothis virescens* pupae. *Comp Biochem Physiol B Biochem Mol Biol*, **2002**, *132*, 505-514.
(76) Gupta, L., J.Y. Noh, Y.H. Jo, S.H. Oh, S. Kumar, M.Y. Noh, Y.S. Lee, S.-J. Cha, S.J. Seo, I. Kim, Y.S. Han, and C. Barillas-Mury, Apolipophorin-III Mediates Antiplasmodial Epithelial Responses in *Anopheles gambiae* (G3) Mosquitoes. *PLoS One*, **2010**, *5*, e15410.
(77) Zdybicka-Barabas, A. and M. Cytrynska, Involvement of apolipophorin III in antibacterial defense of *Galleria mellonella* larvae. *Comp Biochem Physiol B Biochem Mol Biol*, **2011**, *158*, 90-98.
(78) Zdybicka-Barabas, A., S. Staczek, P. Mak, T. Piersiak, K. Skrzypiec, and M. Cytrynska, The effect of *Galleria mellonella* apolipophorin III on yeasts and filamentous fungi. *J Insect Physiol*, **2012**, *58*, 164-177.
(79) Zdybicka-Barabas, A., S. Staczek, P. Mak, K. Skrzypiec, E. Mendyk, and M. Cytrynska, Synergistic action of *Galleria mellonella* apolipophorin III and lysozyme against Gram-negative bacteria. *Biochim Biophys Acta*, **2013**, *1828*, 1449-1456.
(80) Whitten, M.M., I.F. Tew, B.L. Lee, and N.A. Ratcliffe, A novel role for an insect apolipoprotein (apolipophorin III) in beta-1,3-glucan pattern recognition and cellular encapsulation reactions. *J Immunol*, **2004**, *172*, 2177-2185.

(81) Zhang, J., A. Lu, L. Kong, Q. Zhang, and E. Ling, Functional analysis of insect molting fluid proteins on the protection and regulation of ecdysis. *J Biol Chem*, **2014**, *289*, 35891-35906.
(82) Stone, E.F., B.O. Fulton, J.S. Ayres, L.N. Pham, J. Ziauddin, and M.M. Shirasu-Hiza, The circadian clock protein timeless regulates phagocytosis of bacteria in Drosophila. *PLoS Pathog*, **2012**, *8*, e1002445.
(83) Sandiford, S.L., Y. Dong, A. Pike, B.J. Blumberg, A.C. Bahia, and G. Dimopoulos, Cytoplasmic actin is an extracellular insect immune factor which is secreted upon immune challenge and mediates phagocytosis and direct killing of bacteria, and is a Plasmodium Antagonist. *PLoS Pathog*, **2015**, *11*, e1004631.
(84) Vlkova, M., M. Sima, I. Rohousova, T. Kostalova, P. Sumova, V. Volfova, E.L. Jaske, K.D. Barbian, T. Gebre-Michael, A. Hailu, A. Warburg, J.M. Ribeiro, J.G. Valenzuela, R.C. Jochim, and P. Volf, Comparative analysis of salivary gland transcriptomes of *Phlebotomus orientalis* sand flies from endemic and non-endemic foci of visceral leishmaniasis. *PLoS Negl Trop Dis*, **2014**, *8*, e2709.
(85) Gui, Z., C. Hou, T. Liu, G. Qin, M. Li, and B. Jin, Effects of insect viruses and pesticides on glutathione S-transferase activity and gene expression in *Bombyx mori*. *J Econ Entomol*, **2009**, *102*, 1591-1598.
(86) Werren, J.H., S. Richards, C.A. Desjardins, O. Niehuis, J. Gadau, J.K. Colbourne, L.W. Beukeboom, C. Desplan, C.G. Elsik, C.J.P. Grimmelikhuijzen, P. Kitts, J.A. Lynch, T. Murphy, D.C.S.G. Oliveira, C.D. Smith, L.v.d. Zande, K.C. Worley, E.M. Zdobnov, M. Aerts, S. Albert, V.H. Anaya, J.M. Anzola, A.R. Barchuk, S.K. Behura, A.N. Bera, M.R. Berenbaum, R.C. Bertossa, M.M.G. Bitondi, S.R. Bordenstein, P. Bork, E. Bornberg-Bauer, M. Brunain, G. Cazzamali, L. Chaboub, J. Chacko, D. Chavez, C.P. Childers, J.-H. Choi, M.E. Clark, C. Claudianos, R.A. Clinton, A.G. Cree, A.S. Cristino, P.M. Dang, A.C. Darby, D.C. de Graaf, B. Devreese, H.H. Dinh, R. Edwards, N. Elango, E. Elhaik, O. Ermolaeva, J.D. Evans, S. Foret, G.R. Fowler, D. Gerlach, J.D. Gibson, D.G. Gilbert, D. Graur, S. Gründer, D.E. Hagen, Y. Han, F. Hauser, D. Hultmark, H.C. Hunter, G.D.D. Hurst, S.N. Jhangian, H. Jiang, R.M. Johnson, A.K. Jones, T. Junier, T. Kadowaki, A. Kamping, Y. Kapustin, B. Kechavarzi, J. Kim, J. Kim, B. Kiryutin, T. Koevoets, C.L. Kovar, E.V. Kriventseva, R. Kucharski, H. Lee, S.L. Lee, K. Lees, L.R. Lewis, D.W. Loehlin, J.M. Logsdon, J.A. Lopez, R.J. Lozado, D. Maglott, R. Maleszka, A. Mayampurath, D.J. Mazur, M.A. McClure, A.D. Moore, M.B. Morgan, J. Muller, M.C. Munoz-Torres, D.M. Muzny, L.V. Nazareth, S. Neupert, N.B. Nguyen, F.M.F. Nunes, J.G. Oakeshott, G.O. Okwuonu, B.A. Pannebakker, V.R. Pejaver, Z. Peng, S.C. Pratt, R. Predel, L.-L. Pu, H. Ranson, R. Raychoudhury, A. Rechtsteiner, J.G. Reid, M. Riddle, J. Romero-Severson, M. Rosenberg, T.B. Sackton, D.B. Sattelle, H. Schlüns, T. Schmitt, M. Schneider, A. Schüler, A.M. Schurko, D.M. Shuker, Z.L.P. Simões, S. Sinha, Z. Smith, A. Souvorov, A. Springauf, E. Stafflinger, D.E. Stage, M. Stanke, Y. Tanaka, A. Telschow, C. Trent, S. Vattathil, L. Viljakainen, K.W. Wanner, R.M. Waterhouse, J.B. Whitfield, T.E. Wilkes, M. Williamson, J.H. Willis, F. Wolschin, S. Wyder, T. Yamada, S.V. Yi, C.N. Zecher, L. Zhang and R.A. Gibbs, Functional and Evolutionary Insights from the Genomes of Three Parasitoid *Nasonia* Species. *Science*, **2010**, *327*, 343-348.
(87) Dubovskiy, I.M., N.D. Slyamova, V.Y. Kryukov, O.N. Yaroslavtseva, M.V. Levchenko, A.B. Belgibaeva, A. Adilkhankyzy, and V.V. Glupov, The activity of nonspecific esterases and glutathione-S-transferase in *Locusta migratoria* larvae infected with the fungus

Metarhizium anisopliae (Ascomycota, Hypocreales). *Entomological Review*, **2012**, *92*, 27-31.
(88) de Graaf, D.C., M. Brunain, B. Scharlaken, N. Peiren, B. Devreese, D.G. Ebo, W.J. Stevens, C.A. Desjardins, J.H. Werren, and F.J. Jacobs, Two novel proteins expressed by the venom glands of *Apis mellifera* and *Nasonia vitripennis* share an ancient C1q-like domain. *Insect Mol Biol*, **2010**, *19 Suppl 1*, 1-10.
(89) Zug, R. and P. Hammerstein, Wolbachia and the insect immune system: what reactive oxygen species can tell us about the mechanisms of Wolbachia-host interactions. *Front Microbiol*, **2015**, *6*, 1201.
(90) Colinet, D., D. Cazes, M. Belghazi, J.L. Gatti, and M. Poirie, Extracellular superoxide dismutase in insects: characterization, function, and interspecific variation in parasitoid wasp venom. *J Biol Chem*, **2011**, *286*, 40110-40121.
(91) Fernandez, D., A. Valdivia, J. Irazusta, C. Ochoa, and L. Casis, Peptidase activities in human semen. *Peptides*, **2002**, *23*, 461-468.
(92) Lovallo, N. and D.L. Cox-Foster, Alteration in FAD-glucose dehydrogenase activity and hemocyte behavior contribute to initial disruption of *Manduca sexta* immune response to *Cotesia congregata* parasitoids. *J Insect Physiol*, **1999**, *45*, 1037-1048.
(93) Antunez, K., R. Martin-Hernandez, L. Prieto, A. Meana, P. Zunino, and M. Higes, Immune suppression in the honey bee (*Apis mellifera*) following infection by *Nosema ceranae* (Microsporidia). *Environ Microbiol*, **2009**, *11*, 2284-2290.
(94) Kulkarni, M.M., A. Karafova, W. Kamysz, S. Schenkman, R. Pelle, and B.S. McGwire, Secreted trypanosome cyclophilin inactivates lytic insect defense peptides and induces parasite calcineurin activation and infectivity. *J Biol Chem*, **2013**, *288*, 8772-8784.
(95) Rocha, I.F., A. Maller, R. de Cassia Garcia Simao, M.K. Kadowaki, L.F. Angeli Alves, L.F. Huergo, and J.L. da Conceicao Silva, Proteomic profile of hemolymph and detection of induced antimicrobial peptides in response to microbial challenge in *Diatraea saccharalis* (Lepidoptera: Crambidae). *Biochem Biophys Res Commun*, **2016**, *473*, 511-516.
(96) Zhu-Salzman, K., J.-L. Bi, and T.-X. Liu, Molecular strategies of plant defense and insect counter-defense. *Insect Sci*, **2005**, *12*, 3-15.
(97) Elsik, C.G., The pea aphid genome sequence brings theories of insect defense into question. *Genome Biol*, **2010**, *11*, 106.
(98) Zhu, Q., Y. Arakane, R.W. Beeman, K.J. Kramer, and S. Muthukrishnan, Functional specialization among insect chitinase family genes revealed by RNA interference. *Proc Natl Acad Sci U S A*, **2008**, *105*, 6650-6655.
(99) Nation, J.L., *Immunity*, in *Insect Physiology and Biochemistry*, J.L. Nation, Editor. 2008, CRC Press. p. 370.
(100) Khush, R.S., F. Leulier, and B. Lemaitre, Drosophila immunity: two paths to NF-kappaB. *Trends Immunol*, **2001**, *22*, 260-264.
(101) Brennan, C.A. and K.V. Anderson, Drosophila: the genetics of innate immune recognition and response. *Annu Rev Immunol*, **2004**, *22*, 457-483.
(102) Ferrandon, D., J.L. Imler, C. Hetru, and J.A. Hoffmann, The Drosophila systemic immune response: sensing and signalling during bacterial and fungal infections. *Nat Rev Immunol*, **2007**, *7*, 862-874.
(103) Lemaitre, B. and J. Hoffmann, The host defense of *Drosophila melanogaster. Annu Rev Immunol*, **2007**, *25*, 697-743.

(104) Nappi, A.J., E. Vass, F. Frey, and Y. Carton, Superoxide anion generation in Drosophila during melanotic encapsulation of parasites. *Eur J Cell Biol*, **1995**, *68*, 450-456.
(105) Nappi, A.J. and E. Vass, Hydrogen peroxide production in immune-reactive *Drosophila melanogaster*. *J Parasitol*, **1998**, *84*, 1150-1157.
(106) Whitten, M.M. and N.A. Ratcliffe, In vitro superoxide activity in the haemolymph of the West Indian leaf cockroach, *Blaberus discoidalis*. *J Insect Physiol*, **1999**, *45*, 667-675.
(107) Ishii, K., H. Hamamoto, M. Kamimura, and K. Sekimizu, Activation of the silkworm cytokine by bacterial and fungal cell wall components via a reactive oxygen species-triggered mechanism. *J Biol Chem*, **2008**, *283*, 2185-2191.
(108) Bergin, D., E.P. Reeves, J. Renwick, F.B. Wientjes, and K. Kavanagh, Superoxide production in *Galleria mellonella* hemocytes: identification of proteins homologous to the NADPH oxidase complex of human neutrophils. *Infect Immun*, **2005**, *73*, 4161-4170.
(109) Mikonranta, L., J. Mappes, M. Kaukoniitty, and D. Freitak, Insect immunity: oral exposure to a bacterial pathogen elicits free radical response and protects from a recurring infection. *Front Zool*, **2014**, *11*, 23.
(110) Tryselius, Y. and D. Hultmark, Cysteine proteinase 1 (CP1), a cathepsin L-like enzyme expressed in the *Drosophila melanogaster* haemocyte cell line mbn-2. *Insect Mol Biol*, **1997**, *6*, 173-181.
(111) Tua, W.C. and S.C. Lai, Induction of cysteine proteinase in the encapsulation of *Hymenolepis diminuta* eggs in the American cockroach, *Periplaneta americana*. *J Invertebr Pathol*, **2006**, *92*, 73-78.
(112) Ali, M.R. and Y. Kim, Antiviral activity of the inducible humoral immunity and its suppression by eleven BEN family members encoded in *Cotesia plutellae* bracovirus. *Comp Biochem Physiol A Mol Integr Physiol*, **2015**, *179*, 44-53.
(113) Herniou, E.A., E. Huguet, J. Theze, A. Bezier, G. Periquet, and J.M. Drezen, When parasitic wasps hijacked viruses: genomic and functional evolution of polydnaviruses. *Philos Trans R Soc Lond B Biol Sci*, **2013**, *368*, 20130051.
(114) Nazzi, F., S.P. Brown, D. Annoscia, F. Del Piccolo, G. Di Prisco, P. Varricchio, G. Della Vedova, F. Cattonaro, E. Caprio, and F. Pennacchio, Synergistic parasite-pathogen interactions mediated by host immunity can drive the collapse of honeybee colonies. *PLoS Pathog*, **2012**, *8*, e1002735.

Table 1: Summary of seminal fluid proteins (identified and quantitated by iTRAQ), grouped into functional groups. *Fisher's Exact* tests were used for each group to test whether there was a significant over-representation of proteins with changed abundances compared to the full dataset or whether groups of proteins were significantly more or less abundant in a specific group. *ROS: reactive oxygen species.

Functional Group	Full dataset	# proteins significantly changed	More in SF from *Nosema apis* infected drones	Less in SF from *Nosema apis* infected drones	# proteins NOT changed	*Fisher's Exact p*-value: Change vs No change 2-tailed	*Fisher's Exact p*-value: More vs Less
AA metabolism	14	6	0	6	8	ns	ns
Carbohydrate metabolism	17	4	2	2	13	ns	ns
carbon metabolism	5	2	1	1	3	ns	ns
Cell Transport	11	2	1	1	9	ns	ns
DNA replication	5	2	2	0	3	ns	ns
Endocytosis	4	1	0	1	3	ns	ns
Glycolysis	15	10	1	9	5	ns	ns
Immune response	20	15	12	3	5	0.009	0.006
Lipid metabolism	10	6	2	4	4	ns	ns
Nucleotide Metabolism	6	0	0	0	6	0.042	ns
Other	21	3	0	3	18	--	--
Pheromone	8	5	5	0	3	ns	0.014
Phosphorylation	7	4	0	4	3	ns	ns
Protease/peptidase	22	7	4	3	15	ns	ns
Protein biosynthesis	14	6	2	4	8	ns	ns
Protein degradation/ protease	7	1	0	1	6	ns	ns
Respiration	8	5	1	4	3	ns	ns
ROS defence	24	14	6	8	10	ns	ns
Structural	10	5	0	5	5	ns	ns
Transcription/translation	4	0	0	0	4	ns	ns
Unknown	22	8	5	3	14	--	--
Venom, allergen	6	5	2	3	1	0.046	ns

Table 2: Protein abundance differences in the core responsive group, which we initially grouped into extracellular origin, intracellular origin and sperm proteins and secondly further into biological pathways. All protein ratios were statistically different between infected and non-infected males ($p<0.05$). Shown are the BeeBase accession numbers, the gi accessions, gene names, protein names, the protein ratios (Infected/control), TargetP secretory tags (S), Experimental evidence and Biological pathway as determined with GO ontology, KEGG and UniProtKB.

BeeBase	Accession	Gene name	Protein name	*N. apis*/ Control	SD(geo)	p-value	TargetP	Exp. evidence	Biological pathway
Extracellular									
Immune Response									
GB50565	gil328780377	Osi7	Osiris 7	**0.6**	1.46	0.003	S		Immune response
GB54507	gil113194556	ApoL	Apolipophorins-like	**0.7**	1.30	0.000	S		Immune response
GB44311	gil442615249	LOC410075	Actin related protein 1	0.9	1.34	0.000	_	3	Cytoskeletal/immune
GB50000	gil328783425	PEBP1	Phosphatidylethanolamine-binding protein	1.1	1.33	0.001	S		Immune response
GB52829	gil301154217	Cht-Idgf4	Chitinase-like protein Idgf4-like isoform X1	1.1	1.29	0.022	S	U	Immune response
GB41779	gil113194865	CABIN1	Calcineurin-binding protein cabin-1-like	1.1	1.29	0.002	S		Immune response
GB44548	gil110756960	Gld2	Glucose dehydrogenase 2	1.2	1.44	0.008	_	U	Immune response
GB55388	gil328789568	GSS	Glucosinolate sulphatase	1.2	1.28	0.004	S	2	Immune response
GB43580	gil145587043	C1q-VP	C1q-like venom protein	**1.2**	1.27	0.001	S	U	Immune response
GB42887	gil328781229	NPC2	NPC2 homolog	1.1	1.28	0.000	S		Immune/ Lipid metabolism
GB44564	gil328788277	CPC2	NPC2 homolog	1.1	1.18	0.000	S	1	Immune/ Lipid metabolism
GB53565	gil110757656	Cht5	Chitinase 5	**1.2**	1.32	0.000	_	U	Immune response
GB52756	gil194718513	ADPase	Apyrase	**1.3**	1.35	0.000	S		Immune response
GB42053	gil328784263	NPC2	Niemann-Pick disease type C2	**1.5**	1.30	0.000	S		Immune/ Lipid metabolism

GB53911	gil328792803	LOC725725	Peritrophin-1-like isoform X1	**1.9**	1.43	0.000	S		Immune response
Protease/peptidase									
GB40726	gil442633124	MMP25	Matrix metalloproteinase-25-like	0.9	1.32	0.003	_	U	Protease/peptidase
GB42152	gil328776058	Psa	Puromycin sensitive aminopeptidase	0.9	1.32	0.033	_	3	Protease/peptidase
GB48820	gil328793021	AT	Antitrypsin-like	1.1	1.30	0.036	_	U	Protease/peptidase
GB42062	gil328784278	Ppn	Papilin	1.1	1.31	0.024	S		Protease/peptidase
GB43129	gil328791209	JHBP	Haemolymph juvenile hormone binding protein	1.1	1.29	0.041	S		Protease/peptidase
GB42425	gil328785822	APN	Aminopeptidase N-like	**1.3**	1.38	0.027	S		Protease/peptidase
Pheromone									
GB46222	gil90194185	Obp13	Odorant binding protein 13	**1.1**	1.26	0.000	S		Pheromone
GB46223	gil90194187	Obp14	Odorant binding protein 14	1.1	1.28	0.000	_	1	Pheromone
GB46226	gil90194191	Obp17	Odorant binding protein 17	1.1	1.34	0.002	S		Pheromone
GB52324	gil281485643	CSP3	Chemosensory protein 3	1.2	1.37	0.000	S	U	Pheromone
GB50151	gil90194177	Obp9	Odorant binding protein 9	**1.5**	1.33	0.000	S	3	Pheromone
Reactive oxygen species Defence									
GB47478	gil295842196	Gtpx1	Glutathione peroxidase-like 1	**0.8**	1.30	0.003	S		ROS defence
GB43731	gil328782010	Fer1HCH	Ferritin 1 heavy chain homologue	**0.8**	1.35	0.000	S		ROS defence
GB53986	gil328794439	DHRS11	Dehydrogenase/reductase SDR family member 11-like	0.9	1.33	0.000	_	1	ROS defence
GB48634	gil55380579	GPX4	Phospholipid hydroperoxide glutathione peroxidase, mitochondrial isoformX2	1.1	1.26	0.019	S		ROS defence
GB50226	gil28403763	Tsf1	Transferrin 1	1.1	1.33	0.000	S	1, U	ROS defence
GB48478	gil328777681	Mipp1	Multiple inositol polyphosphate phosphatase 1-like	**1.2**	1.54	0.000	S		ROS defence
Venom, allergen									
GB41338	gil113193577	Acph-1	Venom acid phosphatase	**0.7**	1.30	0.000	S	U	Venom, allergen
GB53887	gil328791539	pfam06757	Insect allergen related repeat, Nitrile-specifier detoxification	0.9	1.27	0.004	S		Venom, allergen
GB41857	gil442619505	abd-A	Antigen 5-like protein	1.0	1.30	0.000	S	1, U	Venom, allergen

GB40141	gil442619989	VSVPs	Venom serine carboxypeptidase	1.1	1.33	0.000	S	U	Venom, allergen
Lipid metabolism									
GB53578	gil328791522	Gcase	Glucocerebrosidase	**1.2**	1.15	0.001	S	1	Lipid metabolism
GB42616	gil328781391	HEXB	beta-hexosaminidase subunit beta-like	**1.2**	1.30	0.000	S		Lipid metabolism
Glycolysis									
GB46214	gil328776980	Pgam	Phosphoglycerate mutase	**0.8**	1.33	0.000	_	1	Glycolysis
GB54753	gil328776579	ENO	Enolase-like	**0.8**	1.31	0.000	M	4	Glycolysis
GB54369	gil402765768	Tpi	Triosephosphate isomerase	0.9	1.35	0.000	_	1	Glycolysis
GB46565	gil328786388	PK	Pyruvate kinase	0.9	1.23	0.000	S		Glycolysis
Other									
GB49621	gil328793280	Fuca	Alpha-L-fucosidase	0.9	1.30	0.001	S	1	AA metabolism
GB44223	gil328787886	LAMAN	Lysosomal alpha-mannosidase-like	1.2	1.36	0.000	S	U	Carbohydrate metabolism
GB52775	gil155679	HYAL	hyaluronidase isoform X1	**1.3**	1.35	0.000	S		Carbohydrate metabolism
GB42822	gil328781067	HEXC	Chitooligosaccharidolytic beta-N-acetylglucosaminidase-like	**1.2**	1.15	0.008	S		Carbon metabolism
GB46815	gil328793312	LOC552453	Restin homolog	0.8	1.38	0.000	S		Cytoskeletal
GB40887	gil328793114	Vha26	vacuolar H[+]-ATPase 26kD E subunit	0.8	1.33	0.000	_	3	Phosphorylation
GB40261	gil328775958	LOC552600	Gamma-interferon-inducible lysosomal thiol reductase-like	1.1	1.30	0.003	S		Protein biosynthesis
GB55096	gil328778237	Men	Malic enzyme	0.9	1.24	0.000	_	3	Respiration
GB50076	gil328783259	PAO	Polyamine oxidase	0.9	1.20	0.036	S	3, U	ROS production
GB42560	gil328778941	14-3-3epsilon	14-3-3 protein epsilon	0.9	1.14	0.001	_	4	Signalling
GB46039	gil328782182	TUBA1	Tubulin alpha-1 chain-like	**0.8**	1.40	0.000	S		Structural
GB44133	gil158738	TUBB1	Tubulin beta-1	**0.8**	1.35	0.000	_	**6, 3, 6**	Structural
GB50477	gil328782410	LOC100577527	hypothetical protein	**1.2**	1.44	0.029	S		Unknown

GB50009	gil328783410	LOC408510	hypothetical protein	**1.3**	1.25	0.000	S		Unknown
GB53625	gil328780091	LOC411622	hypothetical protein	**1.4**	1.27	0.000	S		Unknown
Sperm									
Respiration									
GB52700	gil66523769	Mdh	Malate dehydrogenase-like	**0.7**	1.35	0.000	_	2	Respiration
GB47042	gil328789134	SDHA	Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial-like	**0.8**	1.25	0.012	M	2	Respiration
GB48791	gil110760473	Cyc	Cytochrome C, testis-specific-like	**1.1**	1.28	0.000	_	2, 1	Respiration
Amino acid metabolism									
GB41651	gil328786134	Gdh	Glutamate dehydrogenase	**0.8**	1.19	0.023	_	2	AA metabolism
GB50217	gil328783663	OAT	Ornithine aminotransferase, mitochondrial	**0.7**	1.37	0.000	_	2	AA metabolism
GB54446	gil58585145	Argk	Arginine kinase	**0.8**	1.32	0.000	_	2, 1, 6	AA metabolism
GB46120	gil328790671	Got2	Glutamate oxaloacetate transaminase 2	**0.8**	1.30	0.000	_	2	AA metabolism
Glycolysis									
GB40302	gil328775929	Pgk	Phosphoglycerate kinase	0.9	1.38	0.000	_	2, 1	Glycolysis
GB50902	gil328786569	Gapdh	Glyceraldehyde 3-phosphate dehydrogenase	1.1	1.29	0.007	M	2, 4, 5	Glycolysis
Other									
GB48991	gil328785563	HAD	3-hydroxyacyl-CoA-dehydrogenase 2	**0.6**	1.39	0.000	_	2	Lipid metabolism
GB42194	gil820838237	LOC726347	Outer dense fiber protein 2	**0.6**	1.33	0.039		2	Cytoskeletal
GB49449	gil328779893	4-HB-CoAT	4-hydroxybutyrate coenzyme A transferase-like	**0.7**	1.42	0.000	M	2	Phosphorylation
GB49937	gil328783569	Dak	Bifunctional ATP-dependent dihydroxyacetone kinase/FAD-AMP lyase (cyclizing)-like	**0.8**	1.36	0.000	_	2	Carbohydrate metabolism
GB41358	gil62526111	EF1-alpha	Elongation factor 1-alpha F2	0.9	1.22	0.000	_	7, 5,	Protein biosynthesis
GB52724	gil328785041	5NUC	5NUC-like	1.1	1.32	0.000	S	2	DNA replication

GB43689	gil328793030	pfam06757	Insect allergen related repeat, nitrile-specifier detoxification	1.2	1.48	0.000	S	2	Venom, allergen
Intracellular									
Reactive oxygen species Defence									
GB45099	gil509841	SOD2	Superoxide dismutase 2	**0.6**	1.49	0.045	M	5	ROS defence
GB41413	gil283436151	Tpx-4	Thioredoxin peroxidase 4	**0.8**	1.35	0.000	_		ROS defence
GB50595	gil328780311	AR1	Alcohol dehydrogenase [NADP+] A-like	0.9	1.33	0.029	_		ROS defence
GB48904	gil328793352	GST	Glutathione S-transferase-like	0.9	1.30	0.011	_		ROS defence
GB48574	gil328784196	Trx-2	Thioredoxin 2	1.1	1.25	0.040	_		ROS defence
GB47880	gil295849267	Sod1	Cu/Zn Superoxide dismutase 1	1.1	1.34	0.000	_		ROS defence
Structure									
GB49450	gil328779929	TTN1	Titin-like isoform X1	**0.7**	1.38	0.042	M		Structural
GB40389	gil147902613	PRF	Profilin	**0.8**	1.52	0.014	_		Structural
GB51683	gil328790637	AnxB9	Annexin-B9-like	0.9	1.32	0.000	_		Structural
Glycolysis									
GB44426	gil328788393	ADH	Alcohol dehydrogenase [NADP+] B-like	**0.7**	1.34	0.000	_		Glycolysis
GB54370	gil45825128	TPI	Triosephosphate isomerase	0.9	1.35	0.000	_		Glycolysis
GB40735	gil328783053	FBP	Fructose-bisphosphate aldolase-like	0.9	1.42	0.000	_		Glycolysis
GB53086	gil328777674	Fdh	Formaldehyde dehydrogenase	0.9	1.16	0.006	_		Glycolysis
Protein Turnover									
GB40976	gil229892247	Hsp90	Heat shock protein 90	**0.8**	1.45	0.000	_		Protein biosynthesis
GB44398	gil328775858	Prosalpha5	Proteasome alpha 5 subunit	**0.8**	1.24	0.006	_		Protein degradation/protease
GB52641	gil110760197	GLOD4	Glyoxalase domain-containing protein 4	**0.8**	1.27	0.000	M		Protein biosynthesis
GB44541	gil110756956	DDP3	Dipeptidyl peptidase 3-like	0.9	1.23	0.014	_		Protease/peptidase
GB40866	gil229892209	Hsc70-4	Heat shock protein cognate 4	0.9	1.37	0.002	_		Protein biosynthesis
GB47617	gil442632285	PPIL	Peptidyl-prolyl cis-trans isomerase-like	1.1	1.23	0.011	M	1	Protein biosynthesis

Lipid Metabolism								
GB53412	gil328787940	FAS	Fatty acid synthetase	**0.8**	1.21	0.000	_	Lipid metabolism
GB52427	gil328786349	Fim	Fimbrin	0.9	1.20	0.000	_	Lipid metabolism
GB50598	gil328780308	AR	Aldose reductase-like	0.9	1.31	0.034	_	Lipid metabolism
DNA replication								
GB45947	gil328782943	Pcd	Pterin-4a-carbinolamine dehydratase	1.1	1.11	0.018	_	DNA replication
GB50170	gil328783619	(LOC100577783)	DOMON domain-containing protein CG14681-like	1.1	1.27	0.031	S	DNA replication
GB42921	gil328793685	polybromo	Polybromo protein (polybromo)	**1.2**	1.29	0.027	_	DNA replication
Other								
GB47016	gil328789113	CCBL1	Kynurenine-oxoglutarate transaminase 1, mitochondrial-like	**0.8**	1.28	0.014	_	Aa metabolism
GB55205	gil241177036	Mrjp1	Major royal jelly protein 1	**0.8**	1.32	0.016	S	Carbohydrate metabolism
GB48133	gil110758426	L-Ldh	l-lactate dehydrogenase A-like 6A-like	**0.7**	1.23	0.005	_	Carbon metabolism
GB55561	gil328789417	ATOX1	Copper transport protein ATOX1-like	0.9	1.44	0.046	_	Cell Transport
GB41604	gil328786041	Clic	Chloride intracellular channel	1.2	1.30	0.022	_	Cell Transport
GB47960	gil328784400	Moe	Moesin	1.0	1.35	0.014	_	Cytoskeletal
GB45736	gil328791163	Past1	Achaete scute target 1	0.9	1.29	0.008	_	Endocytosis
GB49159	gil328788602	NTF-2	Nuclear transport factor 2-like	**0.7**	1.46	0.003	_	Immune response
GB49448	gil328779891	4-HB-CoAT	4-hydroxybutyrate coenzyme a transferase-like	**0.8**	1.25	0.016	_	Phosphorylation
GB53138	gil328777670	LOC100578006	Inorganic pyrophosphatase-like	**0.8**	1.17	0.005	M	Phosphorylation
GB51042	gil113194865	FH	Fumarate hydratase, mitochondrial-like	**0.8**	1.40	0.025	_	Respiration
GB40157	gil66522467	LOC408421	hypothetical protein	**0.8**	1.18	0.000	_	Unknown
GB53435	gil571551216		chromosome X, complete sequence	**0.8**	1.12	0.031	_	Unknown
GB53925	gil571510377	LOC724993	hypothetical protein	**1.2**	1.27	0.034	S	Unknown

Footnotes: SD(geo) refers to geometric standard deviation. TargetP tag M refers to mitochondrial targeted. Experimental evidence was obtained from the following publications: [1] honey bee SF [25, 27], [2] honey bee sperm [58], [3] mosquito SF [48], [4] bedbugs SF [49], [5] cricket SF [44], [6] fruit fly SF [45, 46], [7] mosquito sperm [48], as well as U- for UniProtKB localisation information.

Table 3: An overview of seminal fluid proteins identified in this study with known links to immune responses in insects or other animals. In order, the first column provides proteins identified, followed by the Bee Base accession, higher or lower abundance in infected males, a brief description of immune functions found in the literature, the organism it was described in and the immune pathway.

Seminal fluid protein	Bee Base	Change	Function as described in the literature	Organism	Immune response pathway
Chitinase 5	GB53565	↑	In *A. gambiae* two group V chitinases, AgBr1 and AgBr2 are secreted into the hemolymph after challenging the mosquitoes by bacterial infections. In honey bees its expression increased in response to *N. ceranae* infection.	*A. gambiae* [62] Honey bee [63]	Toll pathway
Chitinase-like protein Idgf4-like isoform X1	GB52829	↑	Chitinase-like proteins have a role in recognizing *Drosophila* chitin at the wound site and in stimulating the cell growth required for wound healing. It plays a role in defence mechanisms against chitin-containing pathogens.	*Drosophila melanogaster* [64] *G. m. morsitans* [65]	Toll pathway
Peritrophin-1-like isoform X1	GB53911	↑	Peritrophin, one of the components of the peritrophic matrix, was first isolated from the intestine of insects. It is thought to protect insects from invasion of micro-organisms. Peritrophin-1 binds chitin.	*Chinese shrimp* [66]	Toll pathway
Antitrypsin-like	GB48820	↑	Similar SERPINs affect the innate immune system of insects, including the Toll pathway, which is mediated by an extracellular serine proteinase cascade. Inhibitory serine protease inhibitor.	*Manduca sexta* [67] *Drosophila melanogaster* [68]	Toll pathway
Papilin	GB42062	↑	Drosophila papilin Kunitz domains are homologous to the classic Kunitz-type serine protease inhibitors	*Drosophila melanogaster* [57, 68] *Anopheles gambiae* [69]	Toll pathway
Osiris 7	GB50565	↓	Osiris is a peptidoglycan recognition protein that participates in the initial recognition reaction. It is at the front of the kinase cascade that activates Spätzle and the Toll-pathway; activate numerous genes, including	*Drosophila melanogaster* [57] *Plodia interpunctella* [70]	Toll pathway

			those encoding antifungal peptides. Shown to be suppressed in response to DNA virus infection		
NPC2	GB42887	↑	ML superfamily (MD-2- related lipid-recognition), immune signalling pathway. Drosophila melanogaster NPC2 proteins bind bacterial cell wall components and may function in immune signal pathways.	*Drosophila melanogaster* [52, 71] *Anopheles gambiae* [72]	Immune signalling (IMD pathway)
CPC2	GB44564	↑			
Niemann-Pick disease type C2	GB42053	↑			
Calcineurin-binding protein cabin-1-like	GB41779	↑	Calcineurin in *Drosophila* indices Relish-promoted immune responses and functions in haemocytes which promote a tissue-to-tissue signalling cascade required for a robust immune response. Calcineurin contributes to innate immune responses and conveys a nitric-oxide (NO) signal that activates AMP production in *Drosophila* larva. Bacteria fed to the larvae remain confined to the gut but nonetheless induce responses in the fat body.	*Drosophila melanogaster* [73]	JAK/STAT pathway
Phosphatidyl-ethanolamine-binding protein	GB50000	↑	PEBPs are highly protective against bacterial infection in *Drosophila* due to the release of immunity-related proteins in the hemolymph. Results suggest PEBP might be an inhibitor of an immune pathway inhibitor.	*Drosophila melanogaster* [74]	JAK/STAT pathway
	GB49999	↑			
Heat shock protein 90	GB40976	↓	Pea aphids showed upregulation of 15 HSP in response to microbial infection	*Acyrthosiphon pisum* [60]	JAK/STAT Pathway, signalling
Heat shock protein cognate 4	GB40866	↓		*Apis mellifera* [71]	
Matrix metalloproteinase-25-like	GB40726	↓	This protein generates intermediates of defensin, which are also active against pathogens.	Human, mice [53, 56]	Prophenol-oxidase cascade
Apolipophorins-like	GB54507	↓		*Heliothis virescens* [75]	

			Bacterial injection increased the amount of ApoLp-III in moths. Apolipophorin III is involved in immune response against bacteria and fungi. Induce antimicrobial peptides, such as defensin and are capable of multicellular encapsulation.	*Anopheles gambiae* [76] *Galleria mellonella* [77-80]	Prophenol-oxidase cascade
Renin receptor-like	GB47134	↓	Renin receptors were identified in molting fluid of insects. Molting fluid is produced in response to pathogens in the ecdysis, between the layers of the cuticle and functions as immunity protection associated with melanisation.	*Bombyx mori* [81]	Prophenol-oxidase cascade
Circadian clock-controlled protein-like	GB42794	↑	Circadian regulator proteins have complex, pathogen-specific effects on immunity. A protein called TIM regulates resistance to infection in *Drosophila*. Circadian regulation affects phagocytic immune cells at an early stage of specific pathogen recognition and makes a significant contribution to survival of infection.	*Drosophila melanogaster* [82]	Prophenol-oxidase cascade
Actin related protein 1	GB44311	↓	Actin has a cellular immune function in insects, involved in phagocytosis and direct killing of bacteria.	*Anopheles gambiae* [83]	Cellular immune
Apyrase	GB52756	↑	In blood sucking insects, apyrase stops platelet aggregation, but also occurs in many non-blood sucking insects, hence other functions are likely. Apyrase hydrolyses ATP and ADP to AMP and orthophosphate, thereby blocking the physiological signal of damaged cells and tissues. This may be in response to the damage produced by *N. apis* spores.	Bloodsucking insects [84]	Cellular immune
Glutathione S-transferase-like	GB48904	↓	In Silkworm reduced GST expression in response to bacterial infection and exposure to pesticides.	*Bombyx mori* [60, 85] *Nasonia* species [86]	Cellular immune

			Fungal infection of locus larvae enhances activity of GST activity	*Locusta migratoria* [87]	
C1q-like venom protein	GB43580	↑	Venom but not allergen in wasp, C1q VP may be able to inhibit the phenoloxidase pathway, preventing encapsulation. This is a likely function of C1p VP in wasp venom.	*Nasonia vitripennis* [88]	Humoral immune (ROS mediated)
Superoxide dismutase 2	GB45099	↓	ROS are produced by mitochondria in the midgut and secreted to the site of infection in order to raise a humoral immune response. Can be secreted in insect fluids, such as venom. In the mosquito high levels of ROS are required to evoke a reaction to the malaria parasite Plasmodium.	*Anopheles gambiae* [89] *Nasonia* species [86]	Humoral immune (ROS mediated)
Cu/Zn Superoxide dismutase 1	GB47880	↑		*Nasonia vitripennis* [90]	
Aminopeptidase N	GB42425	↑	Aminopetidase N is upregulated in bacteria-challenged Indianmeal larvae, and its changes to the redox state of midgut cells contribute to an enhanced immune state. In human, ectopeptidases such as aminopeptidase N regulate many biological processes and play a prominent role during an immune response.	*Indianmeal Moth* [55]	Humoral immune (ROS mediated)
				Human [91] [54]	
Glucose dehydrogenase 2	GB44548	↑	GDH2 abundance increases in *Manduca sexta* in response to a viral infection and is required in the killing response to fungal invaders. GDH2 is required for the generation of reactive oxygen species (ROS) at infection sites, humoral immune response associated with melanised encapsulation of foreign bodies, and it has as a direct killing effect on fungal pathogens.	*Manduca sexta* [92] Honey bee [93]	Humoral immune (ROS mediated)
Glutathione peroxidase-like 1	GB47478	↓	Down-regulated in infected ovaries of the pill bug.	*Armadillidium vulgare* [57] *Anopheles gambiae* [89]	Humoral immune (ROS mediated)

Thioredoxin peroxidase 4	GB41413	↓	Activated in response to ROS production of *Nomuraea rileyi* infection in Armyworm	*Nasonia vitripenni* [86] *Spodoptera litura*	Humoral immune (ROS mediated)
Transferrin 1	GB50226	↑	Induced by infection, encodes a protein that mediates iron transport.	*Drosophila melanogaster* [64]	Cell Cycle Regulation
Peptidyl-prolyl cis-trans isomerase-like (cyclophilin)	GB47617	↑	Cyclophilin, secreted by the parasite, binds to and inactivates AMPs (such as trialysin) via action at its proline residue. Cyclophilin-trialysin synergistically acts on parasites to activate calcineurin phosphatase signalling, which drives metabolic activation and ATP production leading to enhanced infectivity.	protozoa [94] *Lepidoptera* [95]	AMP inactivation, immune suppression
Glucosinolate sulphatase	GB55388	↑	Glucosinolate sulphatase is an insect counter-attack to toxic glucosinolates from the environment. Its expression is reduced in response to *N. ceranae* infection.	*Plutella xylostella* [96] Honey bee [63]	unspecified

Figure 1

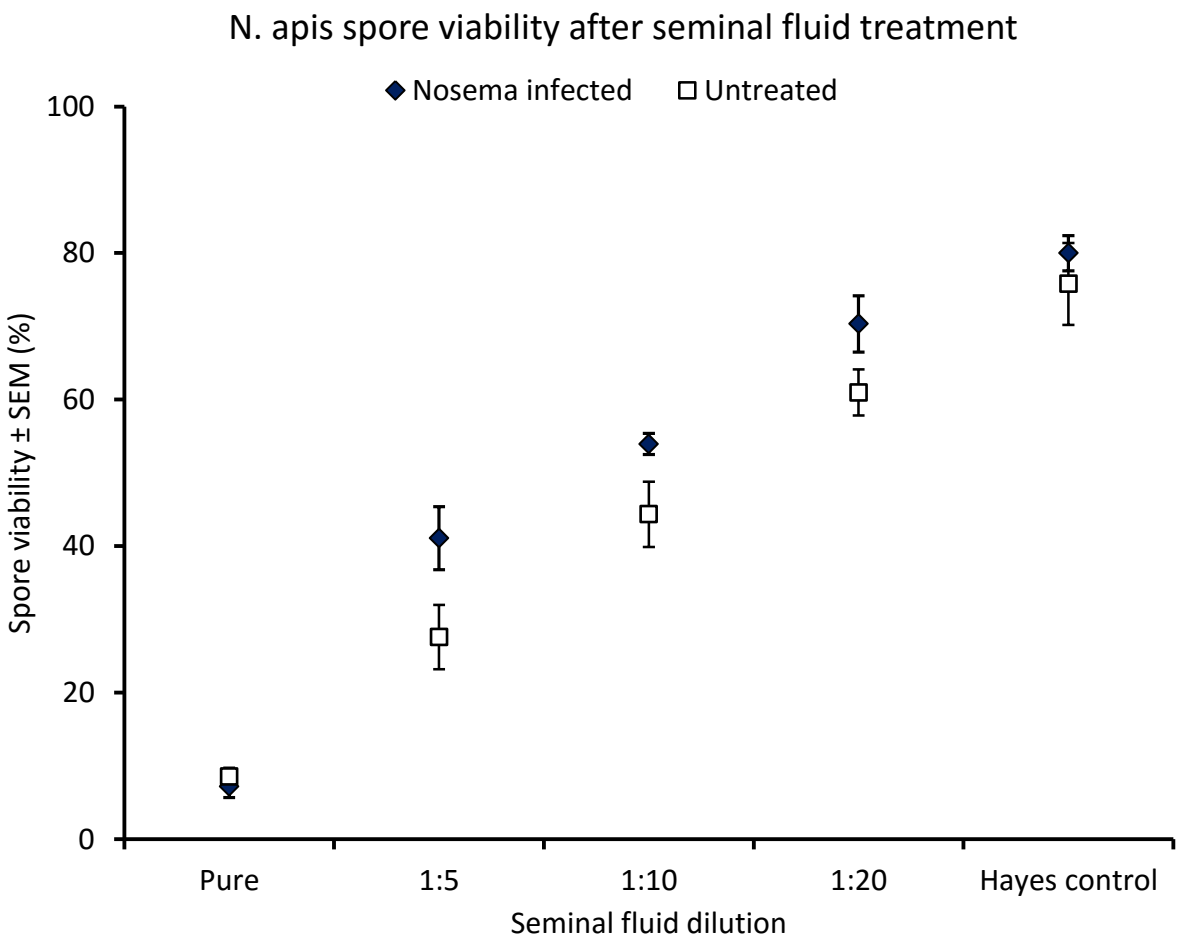
N. apis spore viability after seminal fluid treatment
Nosema infected
Untreated
Spore viability ± SEM (%)
100
80
60
40
20
0
Pure
1:5
1:10
1:20
Hayes control
Seminal fluid dilution

Figure 2: A schematic diagram of the insect immune system.