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Phagocytic events, associated lipid peroxidation and peroxidase activity in hemocytes of silkworm *Bombyx mori* induced by microsporidian infection

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Abstract. Microbial infections induced humoral and cell- mediated immune events in hemocytes. After infection by the microsporidian *Nosema bombycis* in the commercially important silkworm, *Bombyx mori*, hemocytes exhibited deformed nucleus and degranulation of structural granules by exocytosis. Granulocytes showed signs of phagocytosis included formation of microvilli, pseudopodia, engulfment of spores, phagosome formation and membrane porosity. Association of membrane disintegration with infection – induced lipid peroxidation (LPO) was revealed by testing level of malondialdehyde, a byproduct of LPO. LPO activity enhanced significantly ($P < 0.0002$) throughout infection with peak activity in later stages of infection from day 11 accompanied by hemocyte plasma membrane disintegration. Partial increase in LPO activity coupled with increased peroxidase activity recorded in early and mid stages of infection. In later stages, peroxidase activity decreased however LPO increased accompanied by phagocytosis events. In hemocytes, phagocytic events are initiated by activation of genes encoding recognition proteins, aggregation factors and immune-associated proteins. ß-*GRP* expression was down regulated after the infection whereas *CTL-11* enhanced expression on day 10. Humoral lectin enhanced expression on day 6 whereas apolipophorin showed 2.59 fold increase on day 10 after infection. Gene encoding cytoskeletal protein, ß- Actin showed stable enhanced expression throughout infection showing positive correlation ($R^2 = 0.65$) with age after infection. Phagocytosis- associated gene *Eater* from Drosophila showed enhanced heterologous expression. Altogether phagocytic events induced by microsporidian infection are accompanied by increased LPO, decreased peroxidase activity and modulated gene activity in hemocytes of *B. mori.*

Keywords. Phagocytosis, lipid peroxidation, peroxidase activity, *Bombyx mori,* microsporidian infection, hemocytes.

INTRODUCTION

Insects have developed functionally active immune system for survival in the widespread habitat. Insect immune system comprises humoral and cellular responses as well as phenol oxidase cascade culminates in melanisation. Innate immunity components included activation of different pathways such as Toll, IMD and JAK-STAT pathways effecting in production of antimicrobial proteins (Hoffmann 2003; Govind 2008). Cell- mediated responses, facilitated instantaneously by hemocytes against pathogens (Barillas-Mury *et al*. 2000) involved nodulation, encapsulation and phagocytosis depending on size of the pathogen/ parasite (Rosales 2011). Phagocytic response of a cell is evolutionarily conserved from protozoan to mammals (Faurschou and Borregaard 2003; Nazario-Toole and Wu 2017) which involved recognition, binding and ingestion of parasites (Rosales 2011; Kwon *et al*. 2014). Phagocytic receptors evoke different signalling pathways such as Draper activated Draper/Src/Syk/CED-6 pathway (Fullard *et al*. 2009) whereas TEP (thioester containing protein) activated CSD6 pathway (Blandin *et al*. 2004). Activation of signalling leads to cytoskeletal rearrangement, insertion of new membrane for pseudopodia and microvilli formation for parasitic engulfment (Bajno *et al*. 2000). In Drosophila, plasmatocytes and granulocytes act as major phagocytic cells against bacteria (Castillo *et al*. 2006; Lamprou *et al*. 2007) whereas in lepidopterans granulocytes are modified to become phagocytic cells (Ling and Yu 2006; Rebeiro and Brehelin 2006).

Though commercially important silkworm, *Bombyx mori* (Lepidoptera) is domesticated and reared under protected conditions, worms are exposed to pathogenic attack causing major losses to silk industry. In *B. mori*, infection by the obligate intracellular microsporidian parasite *Nosema bombycis* causes devastating disease, pebrine. *N. bombycis* infects either through transovarian transmission or through secondary contamination by feeding contaminated mulberry leaves (Hukuhara 2017). On reaching midgut, spores germinate and inject sporoplasm into midgut epithelial cells (Franzen 2005) where it multiplies and spread to other host tissues including haemolymph and hemocytes. Though pathogens spread through eggs to newly hatched larvae, symptoms or presence of spores could not be identified in initial stages of infection and spores could be identified only after six days of infection (Ma *et al*. 2013).

In *B. mori,* infection by *N. bombycis* suppressed host responses, inhibited melanization events and down regulated immune genes in midgut (Pan *et al*. 2013; Ma *et al*. 2013). However hemocyte- mediated cellular responses against microsporidian infection is not known. In this investigation, cellular, biochemical and molecular immune responses of hemocytes in *B. mori* were revealed showing induction of LPO activity and peroxidase activity in association with phagocytosis events against *N. bombycis* infection.

MATERIALS AND METHODS

Infection and sample collection

B. mori larvae were reared on mulberry (*Morus* sp) leaves under standard rearing conditions of 25 ± 2°C, 70% relative humidity and natural photoregime (13L : 11D). Initially larvae were grown till third instar and separated after third moulting to fourth instar. Day 0 fourth instar larvae were collected and exposed to experimental infection by feeding spores of *N. bombycis* (standard strain: NIK-1s_mys) smeared on mulberry leaf with a dose of 1 x 10^6 spores / larva that induced 50% mortality within the lethal time LT_{50} (Rao *et al*. 2004). Non-infected *B. mori* larvae of the same age group were used as control and reared separately.

In order to analyse hemocyte responses after *N. bombycis* infection, haemolymph samples were collected from control and infected larvae on day 0, 2, 6, 8, 9, 10, 11 and 12 after the infection. Haemolymph was collected by piercing first abdominal proleg using a sterilized needle. Haemolymph of pupae were collected from newly formed pupa (14 days after infection) by puncturing leg impressions on ventral side. The hemocytes were separated by centrifugation at 880 g for 10 min at 4°C. The hemocyte pellets were washed with anticoagulant solution (0.098M NaOH, 0.186M NaCl, 0.017M EDTA, 0.041M Citric acid, pH 4.5 adjusted using NaCl) twice and stored at -80°C for protein analysis. For total RNA extraction, hemocyte pellets were stored in RNA stabilization reagent RNA later (Qiagen).

Light microscopy and transmission electron microscopy (TEM)

Hemocytes of infected and non-infected control larvae were observed under inverted tissue culture microscope (Leica) and the hemocytes and spores were counted using hemocytometer. In order to carryout TEM analysis, hemocyte samples were processed essentially as described earlier (Pradeep *et al*. 2013). Briefly, hemocytes were fixed in 3 % glutaraldehyde upto 24 h before fixation in 1% osmium tetroxide. The samples were dehydrated through alcohol series, brought to acetone and then stained with 2 % uranyl acetate. Using an embedding kit

(Araldite Embed- 812) hemocyte samples were embedded in araldite for 48h. Ultrathin sections (100nm) were cut using Ultramicrotome (Leica –EM UC6) and placed on a copper grid. The ultrathin sections were stained with uranyl acetate and lead citrate and observed under TEM. Ultrastructural variations in the hemocytes (n = 50 each) were observed at 60 kV in a Tecnai G^2 transmission electron microscope attached with Megaview Soft Imaging System and photographed (at the TEM facility in National Institute for Mental Health and Neuro Sciences (NIMHANS), Bangalore, India).

Lipid peroxidation

In order to examine lipid peroxidation in the hemocytes of *B. mori* larva infected with *N. bombycis*, hemocyte samples were collected at different time points after infection along with sample from same aged non-infected control. The assay was performed using EZAssay™ TBARS lipid peroxidation estimation kit following manufacturer's (HiMedia) protocol. Membrane lipids are destructed by phagocytes to form lipid peroxides (Mylonas and Kouretas 1999) which in turn breakdown to form a by-product malondialdehyde (MDA) which is measured by absorbance. The lipid peroxidation was estimated as malondialdehyde (MDA) equivalents (Hodges *et al.* 1999) in a 96 well microtiter plate using absorbance reading at 532 nm in a microtiter plate reader (Multiskan spectrum, Thermo). From 1- 10 µM MDA, a standard curve was obtained by using a slope of standard curve (y = 0.00457+0.00416x) and MDA concentration in the sample was calculated (Yagi 1998; Fatima *et al.* 2011).

Ascorbate peroxidase activity

In order to test whether peroxidase activity varied in hemocytes after the infection, ascorbate peroxidase (PRX) activity was determined as described earlier (Nakano and Asada 1981). Hemocyte pellet was collected from haemolymph at different time points after the infection and then lyzed in Insect cell lysis buffer and quantified the protein (Lowry *et al.* 1951). Briefly, PRX assay was performed using 750 µl 100 mM phosphate buffer (pH- 7.0), 150 µl of 5.0 mM ascorbate and 300 µl 0.5 mM of H_2O_2. The reaction was carried out in 1.5 ml Eppendorf tubes using 300 µl of hemocyte lysate sample containing 100 µg protein. The reaction mixture was transferred to 96 well plate. The rate of decrease in absorbance was read at 290 nm at every one minute interval for ten minutes duration. The enzymatic activity was calculated using a molar extinction coefficient of 2.8 mM-1 cm-1 and the result was expressed as micromoles per minute per milligram of protein (Nakano and Asada 1981).

Hydrogen peroxide activity

In order to test variation in hydrogen peroxide (H_2O_2) level after infection, assay was performed using hemocyte extract in phosphate buffered saline (PBS) buffer (pH 7.1) as described earlier (Velikova *et al.* 2000; Pooja *et al.* 2017). The absorbance was measured at 390 nm in the microplate reader and H_2O_2 content was calculated based on a standard curve.

Total RNA extraction and cDNA synthesis

Total RNA was extracted from hemocytes collected from control and *N. bombycis*- infected *B. mori* larva. Genomic DNA contamination was removed from total RNA by incubation with RNAse free – DNAse I (Takara). Complementary DNA (cDNA) was synthesized from 1 µg total RNA using oligo d(T) primer and M-MuLV (Moloney Murine Leukemia virus) reverse transcriptase using cDNA synthesis kit as per manufacturer's protocol (Primescript; Takara). RT-PCR was performed to analyse semiquantitative expression on 0, 2, 6, 8 and 10 days after infection using gene- specific primers (Table 1) which was validated by qPCR.

Quantitative PCR (qPCR)

qPCR was performed using DyNaMo SYBR GREEN qPCR Master Mix (Thermo - Finzyme) with 0.3X Rox as passive reference dye, on Agilent Stratagene Mx3005P qPCR system. A 25 µl reaction mixture contained 2.5 µl cDNA template, one pmol each of forward and reverse primers and 12.5 µl SYBR Green qPCR master mix (1X) containing 4 mM $MgCl_2$. The thermal program was set as 95°C for 15 min, followed by 40 cycles of 95°C for 30 seconds and at primer- specific annealing temperature (Tm) for a minute (Table 1).The PCR products were resolved by 1.5 % agarose gel electrophoresis and confirmed target-specific amplification. For housekeeping gene, *ribosomal protein* gene was used and fold change in expression relative to calibrator was calculated.

Cloning and sequencing

From *B. mori* genome phagocytosis- associated genes *TEP-1* and *Eater* are not reported. In order to con-

Table 1. Key to the genes and encoding proteins associated with phagocytosis induced in hemocytes of *B. mori* larva after infection with the microsporidian *N. bombycis*.

Gene name	Nucleotide Accession No.	Primer (Left (L) and right (R))	Tm (°C)	Protein Accession No.	Functions	Reference
TEP- 1	AY433751	5'GTGGCTAAGCCCAGTTTCAG3'L 5'AGTCACTCGGAAGCACTCGT3'R	59.4	ABG-0034413	Hemocyte mediated complement like protein, *Plasmodium berghei* binding and mediates killing	Blandin *et al*, 2004
Eater	NM_143276	5'ATAGCCGCTGCTGATGACTC3' 5'TCTTCGATCCGGCAAAAC3'	56.5	Q9VB78	Transmembrane protein, scavenger receptor, bacterial recognition and phagocytosis.	Kocks *et al*, 2005; Chung *et al.*, 2011
βGRP- 2	NM_001043985	5'AATGACACTGTTCGCGTTCC3' 5'TCGCACTCTCGTCTTTGTTG3'	57.3	H9JQ04	Recognition of β- 1, 3 glucan from fungi and mediate cellular responses.	Kim *et al*, 2000 Xiang-Jun Rao *et al* 2014
βGRP- 4	NM_001166142	5'ACCTTGTCGAATCCAGAGGC3' 5'CGGGTCTATTGTTGAAGCCG3'	59.4	Q9NL89	Recognition of β- 1, 3 glucan from fungi and mediate cellular responses.	Kim *et al*, 2000 Xiang-Jun Rao *et al* 2014
CTL- 11	BGIBMGA006623	5'TCTGGTCGGTCGGCTGTATA3' 5'GAGCTGCTCCGCTATGAACT3'	59.4	D2X2F7	Acts as opsonin and increase phagocytosis.	Pendland *et al*, 1988
CTL- 17	NM_001130899	5'ACGTCCTGCATACCGAAAAG3' 5'GCCTCGTCTAACGATTCAGG3'	58.3	Q06FJ6	Acts as opsonin and increase phagocytosis.	Pendland *et al*, 1988
Apolipoph-orin I/II	AB640623	5'TGGCGGATAAATGCTCGTTG3' 5'TCTTCTTCCGCGCAAATCTG3'	57.3	G1UIS8	Act as pattern recognition protein and binds to fungal β- 1, 3 glucan, aid in phagocytosis.	Whitten *et al*. 2004; Barabas and Cytryńska, 2013
Humoral lectin	NP_001104817.1	5'GGCGGTACAACGTTAAGGAG3' 5'AACGAGCACCGACACAAGTA3'	58.3	P98092	Adhesive protein and relates to hemostasis or encapsulation of foreign substances for self-defense.	Kotani *et al*. 1995
Beta Actin A4	NM_001126255	5'ATCCTGCGTCTGGACTTAGC3' 5'AAGACTTCTCGAGGGAGCTG3'	59.4	P84183	Cytoskeletal protein, helps in formation of cellular projections.	May *et al*. 2001; May and Machesk 2001
Ribosomal protein	NP_001037259.1	5'TGGAGCGCCTTACAAACTCT3' 5'GCCAGATTGCTTGGTTGACT3'	57.3	Q5UAN9	House- keeping gene	Lu *et al* 2013

firm heterologous expression of *TEP-1* and *Eater,* mRNA sequence of *TEP-1* of *Aedes aegypti* (Acc No.AY432915.1) and *Eater* of *Drosophila melanogaster* (Acc. No. HM165182) were collected from NCBI database and primers were designed (Primer 3 program) and used for qPCR (Table 1). The amplicons were resolved on 1.2 % agarose gel containing the fluorescent dye ethidium bromide. The *Eater* gene amplified at expected product size. In order to confirm the sequence identity *Eater* amplicon from RT-PCR was purified using a PCR purification kit (QIAquick, Qiagen) and cloned into a vector pJET 1.2/blunt using CloneJet PCR cloning kit (Thermo Scientific) as per manufacturer's protocol. The cloned product was transformed into JM 109 competent cells. Through colony PCR, plasmids were confirmed and the plasmid DNA was isolated using QIAprep spin miniprep kit (Qiagen). Presence of the fragment was confirmed by PCR and sequenced using Sanger method at a facility (Applied Biosystems) available at Eurofins Genomics India Pvt. Ltd., Bangalore, India.

The nucleotide sequence was used to perform BLASTN 2.8.1+ (NCBI) search against non-redundant database. The nucleotide sequence was then translated to protein sequence using Expasy – Translate tool (https://web.expasy.org/translate/). The 3'5' Frame 2 of the translated sequence was used for analysis by BLASTP 2.8.1+ (NCBI) and searched against non-redundant GenBank CDS translations, PDB, SWISSPROT, PIR and PRF.

Statistical analyses

The data were presented as Mean ± SD. Significance of difference between means was evaluated by Students' *t*- test or single factor ANOVA. Correlation between variables was analysed by linear regression (y = a + bx).

Quantitative gene expression was performed using cDNA synthesized from total RNA of hemocytes of control and infected *B. mori* larva, relative to the calibrator using Mx3500P Real Time PCR system (Agilent). Average threshold cycle (Ct) value of transcript expression was calculated from triplicates using ΔΔ Ct method (Livak and Schmittagen 2001) and normalized by housekeeping gene encoding ribosomal protein. Comparative Ct values of the genes were standardized by Ct values for the house keeping gene encoding ribosomal protein. Ct values were standardized with average control value, providing ΔCt value which is standardized to make the average control value '1' (the ΔΔ Ct values) (Gerardo *et al*. 2010). Fold change in gene expression was calculated with reference to the calibrator, which in turn presented down regulated relative quantities as negative values. The data (mean ± SD) denoted is the gene expression induced by infection after eliminating the changes in control.

RESULTS

Organismal variations

Day 0 fourth instar larvae were experimentally infected by feeding spores of *N. bombycis* smeared on mulberry leaf with LD_{50} dose of 1 x 10^6 spores / larva. After the infection, changes were not observed in larval behaviour and activity till day 2. Larval death was recorded from four days after infection. Moulting of infected larvae to fifth instar delayed by 24 h in comparison to control. Infected larvae were smaller in size and showed reduced growth from day 8. Infected larvae initiated cocoon spinning at 24 h after control larvae spun silk. Cocoons of infected larvae were smaller and flimsy with lower silk content. Infected larvae were black and showed melanization under cuticle. Infected pupae were smaller in size and acutely melanized (Fig 1 A-C).

N. bombycis spores were absent in non- infected control larval haemolymph and tissue samples. In *N. bombycis*- infected larvae (n = 30 each), spores were not found till day 6. On day 6, spore count was 0.1 x 10^5 / ml haemolymph. On day 9 the count was 0.95 x 10^5 spores/ ml showing significant ($P< 0.001$) increase in comparison to day 6. Spore count then enhanced significantly ($P < 0.001$) to 4.4 x 10^5 spores / ml on day 10 and to 5.6 x 10^5 spores / ml on day 11 after infection showing the increase in a sigmoid fashion. Within 11 days, average of 60% larval mortality was recorded after the infection.

Cellular variations in hemocytes

Total hemocyte count of control and *N. bombycis*- infected larvae did not vary significantly on day 0 of infection, however significant ($P < 0.005$; ANOVA) decrease observed in later stages (8 to 11 days) of infection. In control, total hemocyte count on day 0 fourth instar larvae was 14.4 x 10^5 cells/ml, which did not vary significantly ($P < 0.1$), on day 0 after infection. The count increased to 16.55 x 10^5 cells/ ml on day 6 however hemocyte count significantly ($P< 0.002$) increased to 17.95 x 10^5 cells/ml after infection. In control, hemocyte count significantly ($P < 0.001$) increased on day 8 to 37.05 x 10^5 cells/ ml whereas after infection it decreased to 32.35 x 10^5 cells/ ml. On day 9 the count was 49.15 x 10^5 cells / ml in control and 45.05 x 10^5 cells / ml after infection. In control on day 10 and 11, mean hemocyte count was 50.4 x 10^5 cells/ ml in comparison to 42.1x 10^5 cells/ ml after infection.

Under light microscope, four types of hemocytes *viz*., granulocytes, plasmatocytes, spherulocytes and oenocytes were observed in addition to the precursor

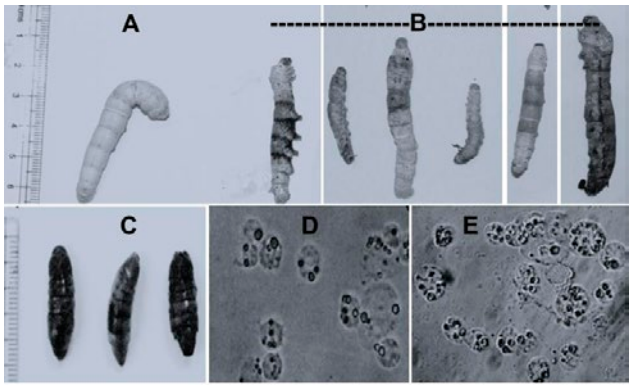

Figure 1. Organismal effects of microsporidian infection on *B. mori* larva: In comparison to control larva (A), infected larvae showed retarded growth and both larvae and pupae melanised (B – C). Control larval hemocytes illustrate clear cytoplasm and less granules (D) whereas infected larval hemocytes were with granulated cytoplasm and few showed degranulation (E).

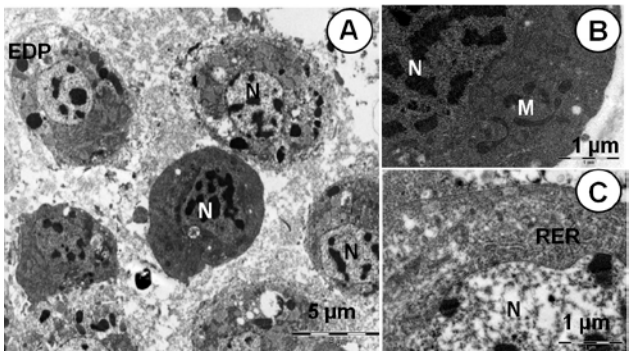

Figure 2. Transmission electron microscopy (TEM) of hemocytes of control fifth instar larvae of *B. mori* (A) showed smooth plasma membrane, clear cytoplasm and cells with few granules and electron dense particles (EDP), nucleus (N) with oval or branched nuclear membrane and evenly distributed chromatin, several mitochondria (M) with developed cristae (B) and rough endoplasmic reticulum (RER) (C).

prohemocytes. In control, hemocytes appeared intact with clear cytoplasm and less granules whereas after infection, cytoplasm becomes granulated on day 6. Many cells ruptured and degranulated from day 8 after infection (Fig.1 D - E).

In order to examine subcellular variations induced by *N. bombycis* infection, infected and control hemocytes were examined under TEM on day 6, 8 and 11 after infection. In hemocytes of control day 6 larvae, cytoplasm was clear. RER and mitochondria with well developed cristae distributed in cytoplasm (Fig. 2 A-C). In plasmatocytes, nuclei were round or ovoid and in granulocytes, smooth or branched. Chromatin was

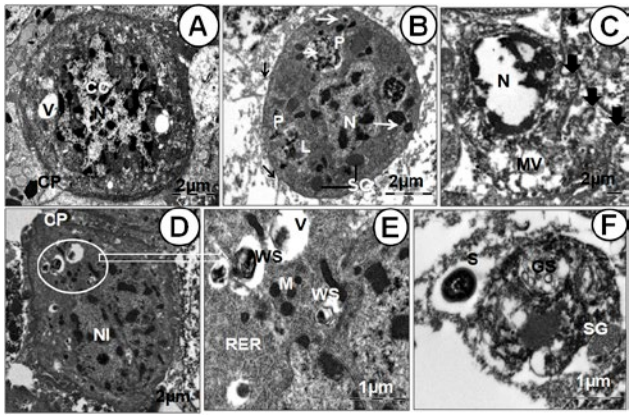

Figure 3. TEM observations on hemocytes of fifth instar larvae of *B. mori* showing subcellular variations after infection with *N. bombycis*: Granulocytes turn phagocytic on day 6 (A-B) showing highly deformed nucleus with condensed chromatin (CC), cellular projections (CP) and few vacuoles (V) in cytoplasm. On day 10 well differentiated pseudopodia (C; black arrow heads), multivesicular body (MV) and phagosomes (P) in association with lysosomes (L) were found. On day 11, hemocytes (D - F) with dense cytoplasmic contents, invaginated nucleus (NI) with condensed chromatin, packets of structured granules (SG), engulfed mature spores (S; white arrows), ghost spores (GS) and whirled sporoplasm (WS) in vacuoles found. Cytoplasm was with RER, mitochondria (M), and vacuoles with cellular remnants (E).

uniformly spread in nucleoplasm. Granulocytes showed presence of few granules and plasmatocytes with few electron dense particles (EDP). Plasma membrane was smooth and with pinocytic vesicles showing active membrane transport.

On day 6 after infection, several packs of structured granules appeared in granulocytes. Large- sized vacuoles occupied major cytoplasmic area**.** Mitochondria increased in number. Many granulocytes featured highly irregular branched nucleus with condensed chromatin. Plasma membrane showed few cellular projections (Fig. 3 A).

On day 8 after infection, granulocytes showed plasma membrane with cytoplasmic extensions, lysosomes and phagosomes with engulfed spores (Fig. 3 B). More granulocytes were with pseudopodia and microvilli (Fig. 3 C). Phagosomes enclosing spores were observed and were associated with lysosomes (Fig. 3 B).

On day 11 after infection, hemocytes showed porous plasma membrane that lost integrity. In few cells, cell membrane completely degenerated. Degranulation by exocytosis observed in close vicinity of spores (Fig. 4 A-B). Many phagocytic granulocytes were observed with cytoplasmic extensions, developed pseudopodia, whirled sporoplasm, engulfed mature spores and ghost spores (Fig. 3 D - F). Rough endoplasmic reticulum (RER) and several mitochondria were observed in

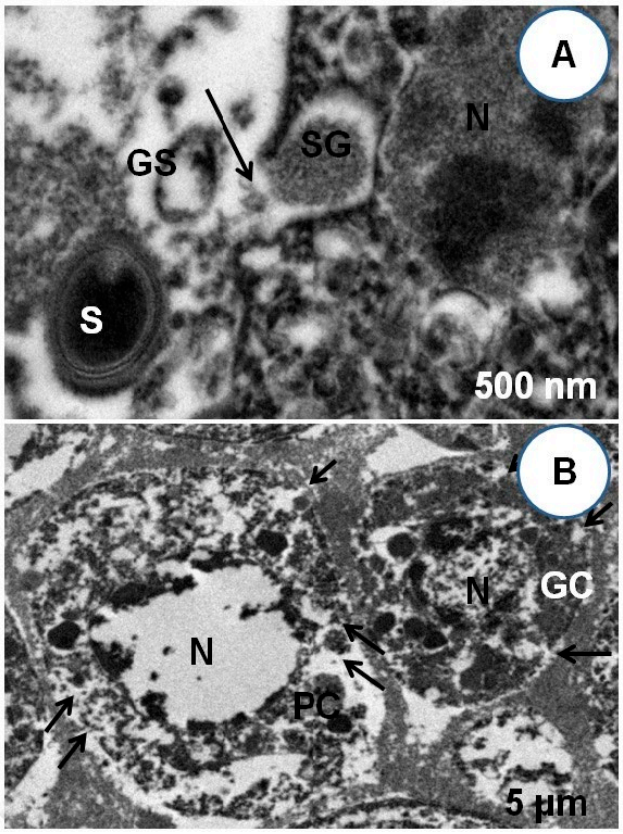

Figure 4. TEM observations on hemocytes of fifth instar larvae of *B. mori* showing subcellular variations after infection with *N. bombycis*. Hemocytes (A) showed degranulation by exocytosis (arrow) to the microsporidian infection locale; (B) Plasmatocyte (PC) and granulocytes (GC) showed membrane disintegration (arrows) after infection with *N. bombycis*. S- spore; GS- ghost spore; SG- structured granules; N- nucleus.

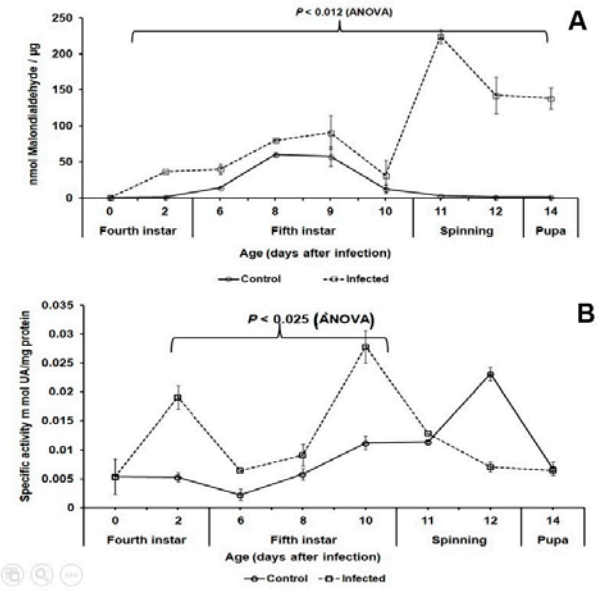

Figure 5 A-B. Lipid peroxidation in hemocytes of *B. mori* larva induced after infection by *N. bombycis* indicated by the variation in malondialdehyde which is a bye-product of lipid peroxidation (A): Lipid peroxidation was at significantly higher level from initial stages of infection and at peak level from day 11 onwards. (B): Variation in ascorbate peroxidase (PRX) activity in hemocytes of *B. mori* larva induced after infection by *N. bombycis*: PRX activity was significantly higher after infection with peak activity on day 2 and 10 followed by decline from day 11 onwards.

dense cytoplasm. Nucleus demorphed, highly intended and showed deep invagination (Fig. 3D). Number of lysosomes increased and located adjacent to spores or fused with vacuoles formed phagosomes.

Lipid peroxidation

In order to examine involvement of lipid peroxidation (LPO) in inducing membrane permeability, LPO was assayed by measuring malondialdehyde (MDA) which is a by-product of lipid peroxidation. After *N. bombycis* infection, MDA levels significantly ($P < 0.012$; ANOVA) increased from day 2 to day 14 with significantly ($P < 0.000004$) larger increase from day 11 indicating increased LPO activity after infection (Fig. 5 A). The increase in LPO activity showed positive correlation with increase in age after infection ($R^2 = 0.58$) as well as number of spores increased exponentially ($y = 0.0003e^{0.5572x}$; $R^2 = 0.80$) with increasing age. In order to verify relation between infection level and LPO increase, correlation analysis was performed which showed significant linear correlation ($R^2 = 0.65$) between increase in spore number and LPO activity.

Ascorbate peroxidase activity

In order to examine change in activity of ascorbate peroxidase (PRX) with increase in infection, PRX activity was measured in hemocytes using ascorbic acid as substrate. PRX activity significantly ($P < 0.025$; ANOVA) enhanced from day 2 to 10 after infection with peak activity on day 10 (Fig. 5 B) whereas control hemocytes showed PRX activity at basic level and increased activity on day 12, during spinning duration. Relation between increase in LPO activity (MDA level) with changes in PRX activity in infected hemocytes was analyzed by correlation- regression which showed positive linear correlation though with low correlation coefficient value ($R^2 = 0.294$) during initial ten days of infection. Notably, from day 11 after infection PRX activity significantly decreased and LPO increased.

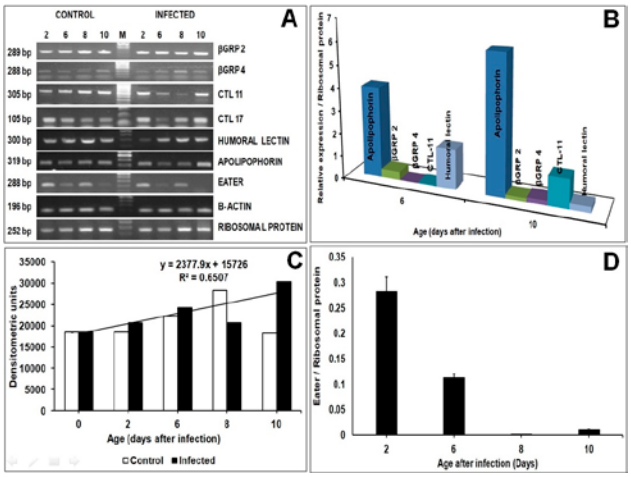

Figure 6 A-D. Modulation in expression of immune genes in hemocytes of *B. mori* larva after infection with *N. bombycis*: (A) RT-PCR profile of gene expression of different genes including cytoskeletal protein gene β- Actin and the house keeping gene encoding ribosomal protein resolved from hemocytes collected from control and infected larvae at 2, 6, 8 and 10 days after infection. M- Massruler DNA marker (Thermo). (B) qPCR showed relative expression of different immune genes in hemocytes at 6 and 10 h after infection. (C) β- Actin, the cytoskeletal protein gene showed gradual increase in expression represented by densitometric units. Increase in expression was correlated with age after infection shown by allometric line. The linear regression formula and correlation coefficient are inserted. (D) Relative expression pattern of the phagocytosis associated gene *Eater like* after standardization with expression of the house keeping gene, ribosomal protein and after elimination of control value.

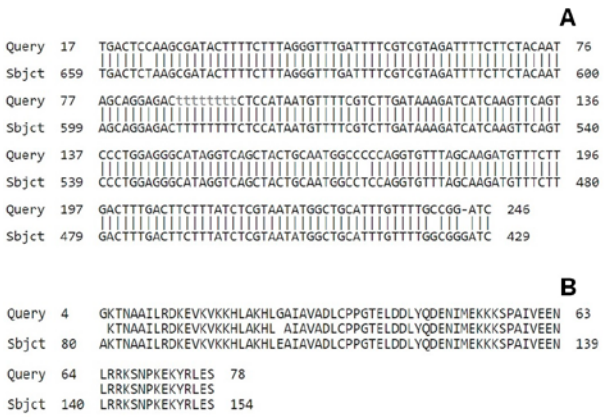

Figure 7. Alignment of nucleotide sequence (A) of amplification product from heterologous expression profile of *Eater* gene of *Drosophila* (query) with nucleotide sequence (subject) of the most similar gene, *B. mori* uncharacterized transcript variant X2 (Accession No. XM_004928120.3) revealed by NCBI-BLASTn analysis, showed 98 % similarity. (B) Protein sequence of the uncharacterized transcript variant (subject) is aligned with translated sequence of the *Eater* like gene (query) showing 97% similarity.

Hydrogen peroxide assay

In order to quantify reactive oxygen species level in hemocyte- phagocytes, level of hydrogen peroxide (H_2O_2) in the hemocytes was tested at 0, 2, 6, 8 and 10 days after the infection using hemocyte lysate extracted in PBS. In hemocytes of *N. bombycis*- infected larvae, H_2O_2 levels did not show significant variation from control (data not provided).

Expression of phagocytosis- associated genes

Humoral immune responses of hemocytes are initiated with recognition of pathogen followed by signalling and effector action. Expression of genes encoding recognition proteins β- glucan recognition proteins (*BGRP2* and *BGRP4*), opsonins C- type lectin (*CTL11 and CTL 17*), hemocyte aggregation factor humoral lectin, phagocytosis enhancer *apolipophorin,* cytoskeletal protein β- *Actin,* hemocyte mediated complement like protein that bind and kill *Plasmodium berghei* in *Aedes aegypti Thioester containing protein* (*TEP-1*) and bacterial phagocytosis associated *Eater* from *Drosophila melanogaster* was analysed by RT- PCR (Table 1) and qPCR (Fig. 6).

RT-PCR profile and qPCR revealed down regulation of βGRP2 and *BGRP4* expression after *N. bombycis* infection. *CTL* genes showed low level of expression in earlier days of *N. bombycis* infection whereas 0.334 fold increase in expression was noticed on 10^{th} day after infection (Fig. 6 B). *Humoral lectin* enhanced relative expression on day 6 (0.79 fold) after infection followed by down regulation (-1.97 fold) on day 10. After infection by *N. bombycis,* apolipophorin showed increase in expression by 1.96 fold on 6^{th} day and by 2.59 fold on 10^{th} day. Expression of β- *actin* showed stable increase from early to late stages of infection with strong positive correlation (R^2 = 0.65) with age after infection (Fig. 6 C).

In the dipterans *A. aegypti* and *D. melanogaster, TEP1* (Blandin *et al.* 2004) and *Eater* (Kocks *et al.* 2005; Juneja and Lazzaro 2010) respectively are closely associated with phagocytosis however these genes are not reported from *B. mori.* Primers derived from *TEP* of *A. aegypti* and *Eater* of *Drosophila* was used for amplification with template cDNA from hemocytes of *B. mori* after infection with *N. bombycis.* In this study *TEP* did not show expression in hemocytes of *B. mori* whereas *Eater* showed enhanced relative expression on day 2 and 6 after infection followed by significant ($P < 0.005$) decrease (Fig. 6 D).

Sequence analysis

In order to confirm presence of *Eater like* gene in *B. mori*, amplification products were ligated, cloned and sequenced. The nucleotide sequence was analysed by NCBI-BLAST. *Eater like* sequence showed 98 % similarity with *B. mori* uncharacterized transcript variant X2 (Accession No. XM_004928120.3) with an expect value 7e-110. The translated sequence of *Eater like* (3'5' frame 2) showed 97 % similarity with translated amino acid sequence of *Bm* uncharacterized protein (*Bm*UCP) *Bm*UCP LOC101736235 isoform X2 (H9JFY7_BOMMO of Uniprot; BGIBMGA008434 of *B. mori)* revealing existence of *Eater like* sequence in *B. mori* genome (Fig. 7). This sequence showed orthology in the lepidopterans *Heliconius melpomene* and *Danus plexippus* with unknown function (EggNOG 4.5.1). However complete sequencing of the gene has to be performed for gene structure confirmation.

DISCUSSION

In the initial stages of microsporidian infection in *B. mori*, spores were not detected microscopically for six days after infection. In the mid and later stages, exponential increase in spore count was recorded. In insects host responses in hemocytes initiated with activation of cell surface receptors and signal transduction (Lamprou *et al* 2007; Tsakas and Marmaras 2010). Hemocytes recognize pathogens entered in larval body with assistance from recognition proteins. The proteins that recognize *Nosema* sporoplasm / spores have not been identified in *B. mori* though microbial recognition proteins such as peptidoglycan recognition proteins (PGRPs) have role in host responses of honey bees against infection by *N. ceranae* through Toll / IMD pathways (Li *et al* 2017). β-GRP (β-1,3-glucan recognition proteins) are recognition proteins in Toll/ Dif pathway (Gobert *et al* 2003) and are associated with detection of bacterial endotoxin in Drosophila (Kim *et al*. 2000) and phenol oxidase activation in *B. mori* (Yoshida *et al*. 1986) indicating multiple role associated with immune reactions. Following recognition, hemocytes initiated cellular immune events such as cell aggregation, nodulation, cytokine release, melanization and encapsulation depend on size of the pathogen / parasite (Lavine and Strand 2002). Similarly, hemocytes initiate phagocytosis against bacteria and fungi particularly against those with size less than five microns (Pech and Strand 1996; Scapigliati and Mazzini 2009). Notably, *N. bombycis* spores infecting *B. mori* larva are of 2.6 to 3.8 microns (breadth x length) (Rao *et al*. 2007) which could be phagocytosed by hemocytes though mechanism of parasite destruction is not clearly known. Phagocytic uptake of KOH treated- or cold storaged *Nosema* spores is found in insect cell line (Cai *et al*. 2012) however phagocytosis of live *Nosema* spores by larval hemocytes *in vivo* had not been reported in *B. mori***.** After *N. bombycis* infection in *B. mori* larva, TEM observation showed symptoms of phagocytosis in granulocytes such as formation of pseudopodia and appearance of phagosomes with lysosomal bags. Spores and ghost spores were observed within phagosomes of hemocytes indicative of lysosomal activity on spores. Phagosomes enclosing spore / meront were found in granulocytes of *B. mori* as noticed in *A. aegypti* after infection by *Plasmodium gallinaceum* (Hillyer *et al*. 2003) and in the coleopteran *Rhynchophorus ferrugineus* infected with the yeast *Saccharomyces cerevisae* (Manachini *et al*. 2011). In the coleopteran flower chafers *Protaetia brevitarsis seulensis,* development of autophagic vacuoles observed in association with phagocytosis by granulocytes indicative of autophagic elimination of pathogens (Kwon *et al*. 2014). After *N. bombycis* infection, granulocytes showed cytoplasmic projections, pseudopodia, microvilli, membrane porosity and disruption as characteristics of phagocytic cells (Castillo *et al*. 2006; Williams 2007). Moreover granulocytes showed degranulation by exocytosis in spore 'locales' indicating active transportation of structural granules to the plasma membrane and degranulation in the site of infection by the spores as observed in mouse models (Dias *et al*. 2018). Hemocytes with extended pseudopodia, cytoplasmic projections and phagosomes were observed in *Anopheles quadrimaculatus* infected with nematode, *Romanomermis culicivorax* (Shamseldean *et al*. 2006), *Culex quinquefasciatus* infected by *Wuchereria bancrofti* (Brayner *et al*. 2007) and in plasmatocytes of the tick *Rhipicephalus sanguineus* infected with *Leishmania infantum* (Feitosa *et al*. 2015). Stable increase in expression of the cytoskeletal protein gene β- actin with age was noticed in hemocytes of *B. mori* after *N. bombycis* infection indicated continuous requirement of actin to redistribute the cytoskeletal protein during formation of pseudopodia and microvilli after *N. bombycis* infection and to meet rearrangement of cytoskeletal proteins for engulfment (Moore *et al*. 1992; Kwon *et al*. 2014). Variation in actin protein content and its critical role was reported in association with formation of pseudopodia in other models also (May and Machesky 2001; Baranov *et al*. 2016).

LPO and peroxidase activity associated with phagocytosis

Infection with *N. bombycis* increased malondialdehyde production in hemocytes revealed increased lipid

peroxidation (LPO). Lipoprotein structure of the membrane is disrupted by LPO, which caused membrane porosity and disintegration in association with phagocytosis. LPO activity significantly increased with age in early and mid stages of infection however it was higher in later stages of infection. Moreover spore count is increased exponentially after six days of infection and lipid peroxidation increased simultaneously showing correlated increase. During lipid peroxidation, carbon-carbon double bonds present in the polyunsaturated fatty acids of plasma membrane are attacked (Yin *et al*. 2011; Wong-ekkabut *et al*. 2007) which is initiated with oxidation of few lipid molecules and subsequently continued as a chain reaction leading to disintegration of cell membrane (Mylonas and Kouretas 1999; Ayala *et al*. 2014). Though infection induced oxidative stress caused lipid peroxidation (Milei *et al*. 2007; Pooja *et al*. 2017), increment in reactive oxygen species (H_2O_2) was not observed in hemocytes of *B. mori* larva after *N. bombycis* infection indicating a direct effect of LPO on hemocyte membrane integrity probably through accumulated toxic products (Clark *et al* 1987). Similar direct impact of lipid peroxidation on tissue damage was reported in liver of the fish *Pimephales promelas* infested by liver trematode *Ornithodiplostomum* sp (Stumbo *et al*. 2012). Accumulation of LPO toxic products could suppress the phagocytic action of hemocytes which defend parasite survival. A possibility for less H_2O_2 content observed in the infected hemocytes might be due to relatively shorter half life induced by its reactivity with biomolecules (Lennicke *et al*. 2015).

In order to protect the cells from peroxidation, enzymatic antioxidant peroxidases are activated (Brigelius-Flohe and Maiorino 2013; Jablonska *et al*. 2015). Ascorbate peroxidase removed lipid peroxides in the lepidopteran *Helicoverpa zea* (Mathews *et al*. 1997) through ascorbate recycling system (Summers and Felton 1993; Krishnan and Kodrik 2006; Lukasik *et al*. 2009). After *N. bombycis* infection, in hemocytes of *B. mori,* peroxidase activity was significantly higher in early and mid- stages of infection which regulated LPO activity to comparatively lower level. In the later stages of infection, peroxidase activity significantly reduced, in contrast, lipid peroxidation increased significantly indicating negative interaction between lipid peroxidation and ascorbate peroxidase activity, corroborating with the negative relation noticed between LPO and peorxidase activity in humans under diseased conditions (McCay *et al*. 1976; Motghare *et al*. 2001). The peroxidase regulation of LPO in association with phagocytic events is unknown in invertebrates after parasitic infection.

Modulation in gene expression after infection

In order to enhance immune reactions after *N. bombycis* infection, genes encoding proteins associated with humoral and cellular immune response were activated before eliciting the host responses (Brown and Gordon 2003; Manachini *et al*. 2011). Notably β-GRP genes did not show significant variability in expression after infection showing an ambiguity in its role in immune reactions against *N. bombycis* infection. On the other hand CTL genes showed upregulated expression on day 6 after infection and its role was suggested to be in spore recognition and signal trasnsduction (Ma *et al*. 2013). *CTL-11 and 17* implicated in opsonising blastospores of the fungus, *Beauveria bassiana* to make fungal spores susceptible to phagocytosis (Pendland *et al*. 1988). Notably, taxonomic position of *N. bombycis* is shifted from protozoan to fungus (Han and Weiss 2017) based on molecular phylogeny. CTL proteins activated during infection by other fungus could be effective during infection by *N. bombycis* probably due to activation of similar immune mechanisms against different species of fungi. Gene encoding hemocyte adhesive factor humoral lectin (Kotani *et al*. 1995) and the phagocytosis enhancer Apolipoprotein III (Whitten *et al*. 2004) enhanced expression after infection. Apolipophorin III together with I/ II help in pattern recognition as well as enhances phagocytic action of hemocytes in insects (Barabas and Cytryńska 2013; Whitten *et al*. 2004).

In the dipterans *Aedes* and *Drosophila, Thioester containing protein (TEP1)* (Blandin *et al*. 2004) and *Eater* (Kocks *et al*. 2005) respectively are associated with phagocytosis however these genes have not reported from *B. mori*. Though *TEP1* did not show expression, *Eater like* showed heterologous expression in hemocytes of *B. mori* larva on day 2 and 6 after infection. Both nucleotide sequence and translated amino acid sequence of *Eater like* amplicon showed 98% similarity with that of an uncharacterized transcript variant from *B. mori* indicating activation of *Eater like* gene in *B. mori* in association with hemocyte- mediated phagocytosis against *N. bombycis*. In *Drosophila*, Eater is a transmembrane protein involved in binding and internalization of bacteria in the phagosomes (Stuart *et al*. 2005; Kocks *et al*. 2005; Chung and Kocks 2011) however role of Eater like protein in *B. mori* immune responses is unknown.

N. bombycis infection induced subcellular variations in hemocytes of *B. mori* including demorphed nucleus, activation of phagocytosis including formation of pseudopodia, microvilli, porous plasma membrane and formation of phagosomes. In addition, lipid peroxidation was increased in hemocytes with increase in age after

infection. Simultaneous increase in peroxidase reduced the LPO activity. In the later stage, peroxidase activity reduced and LPO activity increased showing negative relation. Moreover infection by *N. bombycis* induced modulation of phagocytosis- associated genes where heterologous expression of *Eater* also observed indicating activation of phagocytic events and associated events against *N. bombycis* infection in *B. mori* larva which are potential novel targets for developing new control measures. The protein- based targets could be used to develop antibody- based mechanisms for early detection of microsporidian infection.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

Ayala A, Munoz MF, Arguelles S. 2014. Lipid peroxidation: production, metabolism, and signaling mechanisms of malondialdehyde and 4-hydroxy-2-nonenal. Oxid Med Cell Longev. Article ID 360438; http://dx.doi.org/10.1155/2014/360438

Bajno L, Peng X-R, Schreiber AD, Moore H-P, Trimble WS, Grinstein S. 2000. Focal exocytosis of Vamp3-containing vesicles at sites of phagosome formation. J Cell Biol. 149:697-706.

Baranov MV, Revelo NH, Dingjan I, Maraspini R, Beest M, Honigmann A, Bogaart G. 2016. SWAP70 organizes the actin cytoskeleton and is essential for phagocytosis. Cell Reports 17:1518–1531.

Barillas-Mury C, Wizel B, Han YS. 2000. Mosquito immune responses and malaria transmission: lessons from insect model systems and implications for vertebrate innate immunity and vaccine development. Insect Biochem Mol Biol. 30:429–442.

Blandin S, Shiao SH, Moita LF, Janse CJ, Waters AP, Kafatos FC, Levashina EA. 2004. Complement-like protein TEP1 is a determinant of vectorial capacity in the malaria vector *Anopheles gambiae*. Cell. 116:661–670.

Brayner FA, Araujo HRC, Santos SS, Cavalcanti MGS, Alves LC, Souza JRB, Peixoto CA. 2007. Haemocyte population and ultrastructural changes during the immune response of the mosquito *Culex quinquefasciatus* to microfilariae of *Wuchereria bancrofti*. Med Vet Entomol. 21:112–120.

Brigelius-Flohe R, Maiorino M. 2013. Glutathione peroxidases. Biochim Biophys Acta.1830: 3289–303.

Brown GD, Gordon S. 2003. Fungal β- glucans and mammalian immunity. Immunity. 19:311-315.

Cai S- F, Lu X- M, Qiu H- H, Li M- Q, Feng Z- Z. 2012. Phagocytic uptake of *Nosema bombycis* (Microsporidia) spores by insect cell lines. J Integr Agric. 11:1321-1326.

Castillo JC, Robertson AE, Strand MR. 2006. Characterization of hemocytes from the mosquitoes *Anophles gambiae* and *Aedes aegypti*. Insect Biochem Mol Biol. 36:891-903.

Chung YS, Kocks C. 2011. Recognition of pathogenic microbes by the Drosophila phagocytic pattern recognition receptor eater. J Biol Chem. 286:26524-26532.

Clark IA, Butcher GA, Buffinton GD, Hunt NH, Cowden WB. 1987. Toxicity of certain products of lipid peroxidation to the human malaria parasite *Plasmodium falciparum*. Biochem Pharmacol. 36:543-6.

Dias FF, Amaral KB, Malta KK, Silva TP, Rodrigues GSC *et al* (2018) Identification of piecemeal degranulation and vesicular transport of MBP-1 in liver-infiltrating mouse eosinophils during acute experimental *Schistosoma mansoni* infection. Front. Immunol. 20 https://doi.org/10.3389/fimmu.2018.03019

Fatima MN, Vivek AS, Amreeta D, Uma DP. 2011. Oxidative stress and antioxidant status in primary bone and soft tissue sarcoma. BMC Cancer 11: 382. DOI: 10.1186/1471-2407-11-382.

Faurschou M, Borregaard N. 2003. Neutrophil granules and secretory vesicles in inflammation. Microb Infect. 14:1317–1327.

Feitosa APS, Alves LC, Chaves MM, Veras DL, Silva EM, Alianca ASS, Franca IRS, Goncalves GGA, Lima-Filho JL, Brayner FA. 2015. Hemocytes of *Rhipicephalus sanguineus* (Acari: Ixodidae): Characterization, population abundance, and ultrastructural changes following challenge with *Leishmania infantum*. J Med Entomol 52:1193-1202.

Franzen C. 2005. How do microsporidia invade cells? Folia Parasitol. 52:36-40

Fullard JF, Kale A, Baker NE. 2009. Clearance of apoptotic corpses. J Apoptosis. 14: 1029-1037.

Gerardo NM, Altincicek B, Anselme C, Atamian H, Barribeau SM, de Vos M, Duncan EJ, Evans JD, Gabaldón T, Ghanim M, *et al*. 2010. Immunity and other defenses in pea aphids, *Acyrthosiphon pisum*. Genome Biology 11, R21. doi: 10.1186/gb-2010-11-2-r21.

Gobert V, Gottar M, Matskevich AA, Rutschmann S, Royet J, Belvin M, Hoffmann JA, Ferrandon D. 2003. Dual activation of the *Drosophila* toll pathway by two pattern recognition receptors. Science. 302:2126-30.

Govind S .2008. Innate immunity in *Drosophila*: Pathogens and pathways. Insect Sci. 15: 29–43.

Han B, Weiss LM. 2017. Microsporidia: obligate intracellular pathogens within the fungal kingdom. Microbiol Spectr. 5. doi:10.1128/microbiolspec.FUNK-0018-2016

Hillyer JF, Schmidt SL, Christensen BM. 2003. Hemocyte-mediated phagocytosis and melanization in the mosquito *Armigeres subalbatus* following immune challenge by bacteria. Cell Tissue Res. 313:117–127.

Hodges DM, DeLong JM, Forney CF, Prange RK. 1999. Improving the thiobarbituric acid-reactive-substances assay for estimating lipid peroxidation in plant tissues containing anthocyanin and other interfering compounds. Planta. 207:604–611.

Hoffmann JA. 2003. The immune response of *Drosophila*. Nature 426:33–38.

Hukuhara T. 2017. The epizootiology of pebrine, one of the great scourges of sericulture. J Biochem Biotech 1:1-3.

Jablonska E, Gromadzinska J, Peplonska B, Fendler W, Reszka E, Krol MB, Wieczorek E, Bukowska A, Gresner P, Galicki M, *et al*. 2015. Lipid peroxidation and glutathione peroxidase activity relationship in breast cancer depends on functional polymorphism of GPX1. BMC Cancer. 15:657; DOI 10.1186/s12885-015-1680-4

Juneja P, Lazzaro BP. 2010. Haplotype structure and expression divergence at the *Drosophila* cellular immune gene *eater.* Mol Biol Evol. 27:2284–2299.

Kim YS, Ryu JH, Han SJ, Choi KH, Nam KB, Jang IH, Lemaitre B, Brey PT, Lee WJ. 2000. Gram-negative bacteria-binding protein, a pattern recognition receptor for lipopolysaccharide and beta-1, 3-glucan that mediates the signalling for the induction of innate immune genes in *Drosophila*. J Biol Chem. 275:32721-32727.

Kocks C, Cho JH, Nehme N, Ulvila J, Pearson AM, Meister M, Strom C, Conto SL, Hetru C, Stuart LM, *et al*. 2005. Eater, a Transmembrane protein mediating phagocytosis of bacterial pathogens in *Drosophila.* Cell. 123:335-346.

Kotani E, Yamakawa M, Iwamoto S, Tashiro M, Mori H, Sumida M, Matsubara F, Taniai K, Kadono-Okuda K, Kato Y, Mori M. 1995. Cloning and expression of the gene of hemocytin, an insect humoral lectin which is homologous with the mammalian von Willebrand factor. Biochem Biophys Acta. 1260:245-58.

Krishnan N, Kodrik D. 2006. Antioxidant enzymes in *Spodoptera littoralis* (Boisduval): Are they enhanced to protect gut tissues during oxidative stress? J. Insect Physiol. 52: 11–20.

Kwon H, Bang K, Cho S. 2014. Characterization of the hemocytes in larvae of *Protaetia brevitarsis seulensis*: Involvement of granulocyte- mediated phagocytosis. PLoS ONE. 9: e103620.

Lamprou I, Mamali I, Dallas K, Fertakis V, Lampropoulou M, Marmaras VJ. 2007. Distinct signalling pathways promote phagocytosis of bacteria, latex beads and lipopolysaccharide in medfly hemocytes. Immunology. 121: 314-327.

Lavine MD, Strand MR. 2002. Insect hemocytes and their role in immunity. Insect Biochem Mol Biol. 32: 1295–1309.

Lennicke C, Rahn J, Lichtenfels R, Wessjohann LA, Seliger B. 2015. Hydrogen peroxide – production, fate and role in redox signalling of tumor cells. Cell Commun. Signal. 13:39. DOI 10.1186/s12964-015-0118-6

Li W, Evans JD, Li J, Su S, Hamilton M, Chen Y. 2017. Spore load and immune response of honey bees naturally infected by *Nosema ceranae*. Parasitol Res. 116:3265-3274.

Ling E, Yu Q- X. 2006. Hemocytes from the tobacco hornworm *Manduca sexta*have distinct functions in phagocytosis of foreign particles and self dead cells. Dev Comp Immunol. 30:301-309.

Livak, KJ, Schmittagen TD. (2001) Analysis of relative gene expression data using real time quantitative PCR and the 2–ΔΔ CT method. Methods 25:402–408.

Lowry OH, Rosebrough NJ., Farr AL, Randall RJ. 1951. Protein measurement with the folin-phenol reagent. J. Biol. Chem. 193:265–275.

Lukasik I, Golawska S, Wójcicka A. 2009. Antioxidant defense mechanisms of cereal aphids based on ascorbate and ascorbate peroxidase. Biologia. 64:994-998.

Ma Z, Li C, Pan G, Li Z, Han B, Xu J, Lan X, Chen J, Yang D, Chen Q, Sang Q, Ji X, Li T, Long M, Zhou Z. 2013. Genome-wide transcriptional response of silkworm *(Bombyx mori)* to infection by the microsporidian *Nosema bombycis.* PLoS ONE 8(12): e84137. doi:10.1371/journal.pone.0084137.

Manachini B, Arizza V, Parrinello D, Parrinello N. 2011. Hemocytes of *Rhynchophorus ferrugineus* (Olivier) (Coleoptera: Curculionidae) and their response to *Saccharomyces cerevisiae* and *Bacillus thuringiensis*. J Invertebr Pathol. 106:360-365.

Mathews MC, Summers CB, Felton GW. 1997. Ascorbate peroxidase: A novel antioxidant enzyme in insects. Arch Insect Biochem Physiol. 34:57-68

May RC, Machesky LM. 2001. Phagocytosis and the actin cytoskeleton**.** J Cell Sci. 114:1061-107.

McCay PB, Gibson DD, Fong K-A, Hornbrook KR (1976) Effect of glutathione peroxidase activity on lipid peroxidation in biological membranes. Biochim Biophys Acta - Lipids Lipid Metab. 431:459-468.

Milei J, Forcada P, Fraga CG, Grana DR *et al.* 2007. Relationship between oxidative stress, lipid peroxidation and ultrastructural damage in patients with coronary artery disease undergoing cardioplegic arrest/reperfusion. Cardiovasc Res. 73:710–719.

Moore CA, Beckmann N, Morse MP. 1992. Cytoskeletal structure of diseased and normal hemocytes of *Mya arenaria.* J Invertebr Pathol. 60:141-147.

Motghare KS, Bhutey A, Murrhar BB, Gupta M, Meshram AM, Balsubramanium Y. 2001. Lipid peroxidation and glutathione peroxidase in ischemic heart disease. Indian J Clin Biochem. 16:213–215.

Mylonas C, Kouretas D. 1999. Lipid peroxidation and tissue damage. In Vivo 13:295-309.

Nakano Y, Asada K. 1981. Hydrogen peroxide is scavenged by ascorbate- specific peroxidase in spinach chloroplasts. Plant Cell Physiol. 22:867-880.

Nazario-Toole AE, Wu LP. 2017. Phagocytosis in insect immunity. Adv Insect Physiol. 52:35-82.

Pan G, Xu J, Li T, Xia Q, Liu S-L, Zhang G, Li S, Li C, Liu H, Yang L, *et al.* 2013. Comparative genomics of parasitic silkworm microsporidia reveal an association between genome expansion and host adaptation. BMC Genomics. 14:186.

Pech LL, Strand MR. 1996. Granular cells are required for encapsulation of foreign targets by insect haemocytes. J Cell Sci. 109:2053-2060.

Pendland JC, Heath MA, Boucias DG. 1988. Function of a galactose- binding lectin from *Spodoptera exigua* larval haemolymph: Opsonization of blastospores from entomogenous hyphomycetes. J Insect Physiol. 34:533-540.

Pooja M, Pradeep ANR, Hungund SP, Chandrashekhar S, Kangayam MP, Awasthi AK, Trivedy K. 2017. Oxidative stress and cytotoxicity elicited lipid peroxidation in hemocytes of *Bombyx mori* larva infested with dipteran parasitoid, *Exorista bombycis.* Acta Parasitol. 62:1230-2821

Pradeep AR, Anitha J, Awasthi AK, Babu MA, Murthy NG, Arun HK, Sagar C, Rao GC, Vijayaprakash NB. 2013. Activation of autophagic programmed cell death and innate immune gene expression reveals immuno-competence of integumental epithelium in *Bombyx mori* infected by a dipteran parasitoid. Cell Tissue Res, 352**:**371–385.

Rao SN, Muthulakshmi M, Kanginakudru S, Nagaraju J. 2004. Phylogenetic relationships of three new microsporidian isolates from the silkworm, *Bombyx mori*. J Invertebr Pathol. 86:87-95.

Rao SN, Nath BS, Bhuvaneswari G, Urs SR. 2007. Genetic diversity and phylogenetic relationships among microsporidia infecting the silkworm, *Bombyx mori*, using random amplification of polymorphic DNA: Morphological and ultrastructural characterization. J Invertebr Pathol. 96:193–204.

Rebeiro C, Brehelin M. 2006. Insect haemocytes: What type of cell is that? J Insect Physiol. 52:417-429.

Rosales C. 2011. Phagocytosis, a cellular immune response in insects. Invertebrate Surviv J. 8:109-131.

Scapigliati G, Mazzini M 1994. *In vivo* and *in vitro* phagocytosis by hemocytes of the stick insect *Bacillus rossius*, Boll Zool. 61:115-120.

Shamseldean MM, Platzer EG, Gaugler R. 2006. Ultrastructure of the Immune Responses of *Anopheles quadrimaculatus* to *Romanomermis culicivorax* (Nematoda: Mermithidae) Infection. Nematropica. 36:243-249.

Stuart LM, Stehle T, Hoffmann JA, Reichhart JM, Ferrandon D, Rämet M, Ezekowitz RAB. 2005. Eater, a transmembrane protein mediating phagocytosis of bacterial pathogens in *Drosophila.* Cell. 123:335–346.

Stumbo AD, Goater CP, Hontela A. 2012. Parasite-induced oxidative stress in liver tissue of fathead minnows exposed to trematode cercariae. Parasitology. 139:1666-71.

Summers CB, Felton GW. 1993. Antioxidant role of dehydroascorbic acid reductase in insects. Biochim. Biophys. Acta 1156:235–238.

Tsakas S, Marmaras VJ. 2010. Insect immunity and its signalling: an overview. Invertebrate Surviv J. 7:228-238.

Velikova V, Yordanov I, Edreva A. 2000. Oxidative stress and some antioxidant system in acid rain treated bean plants: protective role of exogenous polyamines. Plant Science 151:59–66.

Whitten MMA, Tew IF, Lee BL, Ratcliffe NA. 2004. A novel role for an insect apolipoprotein (Apolipophorin III) in β-1,3- glucan pattern recognition and cellular encapsulation reactions. J Immunol. 172:2177-2185.

Williams MJ. 2007. Drosophila hemopoiesis and cellular immunity. J Immunol. 178:4711-4716.

Wong-ekkabut J., Xu Z., Wannapong T., Tang I-M., Tieleman D.P.,Monticelli L. 2007. Effect of lipid peroxidation on the properties of lipid bilayers: a molecular dynamics study. Biophysical Journal, 93:4225–4236. DOI: 10.1529/biophysj.107.112565

Yagi K. 1998. Simple assay for the level of total lipid peroxides in serum or plasma. Methods in Molecular Biology 108:101–106. DOI:10.1385/0-89603-472-0:101

Yin H., Xu L., Porter N.A. 2011. Free radical lipid peroxidation: mechanisms and analysis. *Chemical Reviews*, 111:5944–5972. DOI: 10.1021/cr200084z

Yoshida H, Ochiai M, Ashida M. 1986. β- 1,3- glucan receptor and peptidoglycan receptor are present as separate entities within insect pro-phenoloxidase activating system. Biochem Biophys Res Commun. 141:1177-1184.