**Research Article**

# IMMUNE SUPPRESSION BEING THE CAUSE FOR ESTABLISHMENT OF *NOSEMA BOMBYCIS* PARASITISM IN THE SILKWORM *BOMBYX MORI*

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**ABSTRACT**

**Pebrine is a devastating disease caused by obligate intracellular microsporidian parasite *Nosema bombycis* in the silkworm *Bombyx mori*. Though organismal observations after infection are reported, larval immune responses are seldom analysed. The infection reduced hemocyte number and induced subcellular variations like membrane porosity, lysis and disintegration. On infection, hemocytes recognize pathogens through recognition proteins that trigger defense reactions. However after *N. bombycis* infection, expression of immune genes pertaining to recognition, Toll signalling and melanization pathways were suppressed, revealed by RT-PCR and Real Time analysis. Recognition genes *β-GRP 2* and *β-GRP 4* were inactivated thereby delayed or inhibited recognition. Toll activator *Spatzle* expression was suppressed and titer reduced early causing delayed binding with 'Toll'. Gene encoding *Cactus* expression increased whereas expression of NF kappa B transcription factors Dorsal and Relish reduced in early stage of infection. Immediately after infection melanisation pathway genes *PPAE* and *PPO2* activated, subsequently suppressed expression causing loss of 'melanization' in later stage of infection. Paralytic peptide titer that induced melaniztion, reduced in early stage of infection. Delayed recognition and signalling activities allowed distraction of *N. bombycis* from primary immune reactions of the host leading to successful establishment of parasitic survival. Further due to lack of melanisation in later stages, *N. bombycis* surmount the host – defenses and establish itself in infected larvae of *B. mori*. Different immune pathway components suppressed by the microsporidian infection could be used as targets for antibody – based early detection mechanisms.**

## INTRODUCTION

Insect's survival under wild and domesticated environments is realized by the defense reactions including host responses and immune reactions against pathogens. Insect immune system comprises non-specific melanisation reactions, humoral immune responses and cell- mediated immune responses (Krautz *et al*., 2014**)**. Humoral responses include recognition of pathogens, signalling and activation of transcription factors and production of antimicrobial proteins (AMP). The AMPs are mainly produced in fat body however midgut and integumental epithelium are also known to induce immune responses (Pradeep *et al.,* 2012; Ma *et al*., 2013). In insects the cellular responses are facilitated by hemocytes which are elicited after recognition of the foreign body (Barillas-Mury *et al*., 2000). Cellular responses included nodulation, encapsulation and phagocytosis depending on the size of the pathogen (Satyavati *et al*., 2014). In addition, upon infection, phenol oxidase cascade is activated resulting in melanisation which is associated with cellular responses as well as pathogen induced wound (Dimopoulos *et al.,* 2001).

The mulberry silkworm *Bombyx mori* L. is domesticated over 5000 years and the domestication made them susceptible to different pathogens. Prevalence of several diseases in silkworm during course of rearing causes major revenue loss to silk industry. Among the various diseases caused in *B. mori*, pebrine caused by microsporidian *Nosema bombycis* is destructive.

Upon infection, immune responses are activated in different insects (Vilcinskas, 2010; Mareno-Gracia *et al.,* 2013) whereas the pathogen surmounts the host responses for its

survival. Though hymenopteran endoparasitoids inject venomous compounds to host larvae to suppress host defense responses (Goecks *et al.,* 2013), pathogenic impact on host immune responses are comparatively less known. Few studies on suppression of host responses by pathogens included bacterial / viral / fungal infection on lepidopteran larvae (Park *et al.,* 2009; Fallon *et al*., 2011; Jakubowska *et al*., 2013; Thakur *et al.,* 2014). In the honey bee, antimicrobial peptide expression was reduced by *N. ceranae* whereas in midgut of *B. mori*, *N. bombycis* infection decreased expression level of genes encoding recognition proteins in the initial stage of infection followed by enhanced expression (Ma *et al.,* 2013).

It is important to note that several isolates of *N. bombycis* are identified within India and other countries. Different isolates of *Nosema* showed different infection rate (Kawarabata, 2003) indicating differential immunity against each pathogen. Hence it is essential to understand the innate immunity changes in host silkworm against Indian isolates of *N. bombycis*. In this study, we have analysed expression profile of immune genes in hemocytes of *B. mori* after infection by the standard isolate of microsporidian *N. bombycis* to reveal the influence of pathogen on innate immunity.

## MATERIALS AND METHODS

### Infection and sample collection

*B. mori* larvae were collected on day 0 fourth instar**.** Each larva was infected with microsporidian *N. bombycis* (standard strain NIK-1s_mys) by feeding spores smeared on mulberry leaf ($LD_{50}$ = 1 x $10^6$ spores) (Rao *et al.,* 2004). Non- infected parallel aged larvae were used as control. Haemolymph samples were collected from control and infected larvae on day 0, 2, 4, 6, 8, 9, 10 and day 11 after infection. Hemocytes were separated from haemolymph plasma by centrifugation at 880 g for 10min at $4^0$C. Hemocytes 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 -$80^0$C for protein analysis. For total RNA extraction, hemocyte pellets were stored in RNA later solution (Qiagen) and extracted using RNAiso plus (Takara).

### Transmission Electron Microscopy (TEM)

Hemocyte samples were processed as described earlier (Pradeep *et al*., 2012). Briefly, hemocytes were fixed in 3% glutaraldehyde upto 24h before fixation in 1% osmium tetraoxide. After dehydrating with alcohol, hemocyte samples were stained with 2% uranyl acetate. Using an embedding kit (AralditeEmbed- 812) hemocyte samples were embedded in araldite for 48h. Ultrathin sections (100nm) were cut with Ultramicrotome (Leica –EM UC6) and placed on a copper grid. Uranyl acetate and lead citrate were used to stain the ultrathin sections of sample. Ultrastructural variations in the hemocytes (*n* = 50 each) were observed at 60 kV in a Tecnai $G^2$ transmission electron microscope (facility with NIMHANS, Bangalore, India) attached with Mega view Soft Imaging System and photographed.

### Cytokine titre determination by ELISA

For protein extraction, hemocytes were lysed with lysis buffer (50mM HEPES, 75mM NaCl, 1mM EDTA, 1mM DTT) containing 0.1% Triton X-100 (Sigma) and 10X protease inhibitor (P2714, Sigma).

In order to examine titre variation of the insect cytokines, paralytic peptide and spatzle in hemocytes of *B. mori* after *N. bombycis* infection, indirect ELISA (Crowther, 1995) was performed using specific primary antibodies raised in rabbit.

### Total RNA extraction and cDNA synthesis

Total RNA was extracted from hemocytes collected from control and *N. bombycis* infected larvae of *B. mori*. Genomic DNA contamination was removed by treating with RNAse free – DNAse I (Takara). Complementary DNA (cDNA) was synthesized from 1µg total RNA using oligo d(T) primer and M-MuLV (Moloney Murine Leukemia virus) reverse transcriptase using the cDNA synthesis kit as per manufacturer's protocol (Primescript; Takara).

### Quantitative PCR

Quantitative PCR was performed using DyNaMo SYBR GREEN qPCR Master Mix (Thermo - Finzyme) with 0.3X Rox II 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 the 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 for a minute. For housekeeping gene, gene encoding ribosomal protein was used and fold change in expression, relative to calibrator was calculated.

### Statistical analyses

The data were presented as Mean ± SD. The significance in difference between means was evaluated by Students' *t*- test and ANOVA.

Quantitation of gene expression in the infected hemocytes was performed relative to the calibrator using Mx3500P qPCR software (Agilent). ΔΔ Ct method (Livak and Schmittagen, 2001) was used to calculate average threshold cycle (Ct) value of transcript expression from the triplicates and normalized by the house-keeping gene encoding ribosomal protein. Comparative Ct values of immune related genes were standardized by Ct values for the control. Ct values were standardized relative to average value for the control, providing the delta Ct value, and these were the values standardized to make the average control value '1' (the ΔΔ Ct values) (Gerardo *et al.,* 2010). The data (mean ± SD) signified is the gene expression induced after infection, eliminating the changes in control. This calculation permitted the display of downregulated relative

quantities as negative values when fold change in gene expression was considered.

## RESULTS AND DISCUSSION

Host *B. mori* larvae evoke immune responses to defend themselves against infections of *N. bombycis* whereas the parasite acts to suppress the defense reactions to support survival in host. *N. bombycis* evolves various strategies for host immune suppression and succeeds to establish them inside *B. mori.* In order to detect parasitic strategies for its survival immune responses at protein and genic level are analysed.

### Hemocyte - responses

Light microscopy of control hemocytes showed clear cytoplasm and normal nucleus whereas in infected larvae many hemocytes granulated (Fig. 1A and B). TEM of control hemocytes showed normal cells with well defined plasma membrane, clear cytoplasm with few granules and intact oval or branched nucleus (Fig. 1C). After *N. bombycis* infection, dividing meronts were observed in hemocytes showing robust infection. Plasma membrane was disrupted and integrity was lost. Cytoplasm was dense with cellular remnants and nucleoplasm with less chromatin (Fig.1D). Hemocytes are known to attack foreign invaders by aggregation, nodulation or encapsulation (Alves e Silva *et al.,* 2013) depending on size of the pathogen (Rosales, 2011). *N. bombycis* spores are 2.6 to 3.8 micron length x breadth (Rao *et al*., 2007) and are possible to be engulfed by hemocytic phagocytosis or could be nodulated / encapsulated as noticed after bacterial infections (Rosales, 2011).

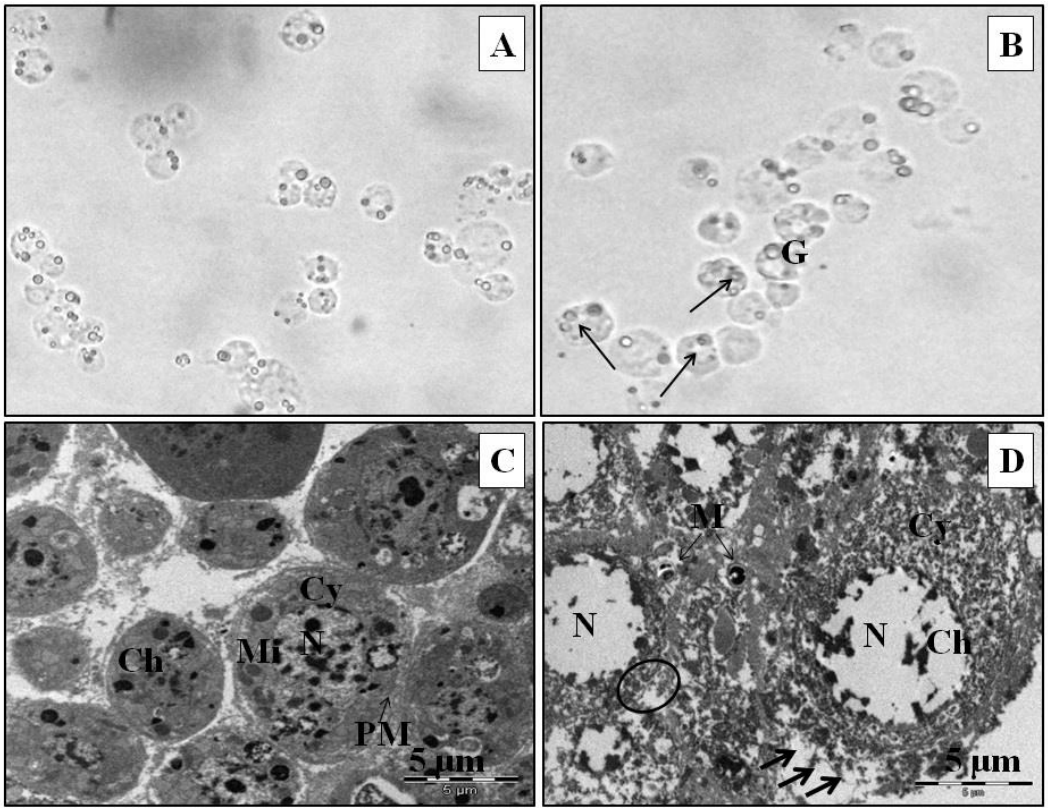


**Fig. 1. A-B:** Light microscopic observations; Control **(A)** larval hemocytes of *B. mori* with clear cytoplasm and intact nucleus. Hemocytes after *N. bombycis* infection **(B)** showing granulated (G) cytoplasm and infected *N. bombycis* spores (black arrows) (400 x). TEM observations of fifth instar control **(C)** larval hemocytes with clear cytoplasm, intact plasma membrane (PM), mitochondria (Mi) and intact oval nucleus (N) with distributed chromatin (Ch). Ultrastructural variations in hemocytes after *N. bombycis* infection **(D)** showed porous plasma membrane (black circle), plasma membrane disintegration (bold black arrows), less chromatin (Ch) and cellular remnants filled- cytoplasm. Meronts (M) of *N. bombycis* were observed in cytoplasm.

### Melanization reactions

The cell- mediated immunity supports activation of innate immunity in insects (King and Hillyer, 2012). However in early and mid-stage of infection, insect cytokine and growth blocking peptide 'paralytic peptide (PP)' titer did not vary significantly ($P < 0.1$) whereas on day 10, titer increased significantly ($P < 0.005$) followed by decrease in comparison to control (Fig. 2A). PP is known to activate the

melanisation enzyme DOPA decarboxylase (DDC) and increased melanisation after infection in lepidopterans (Noguchi *et al*., 2003) however after *N. bombycis* infection, PP level did not vary, rather decreased than control.

In order to examine the expression profile of genes encoding components of melanisation pathways, RT-PCR and qPCR were performed using candidate gene approach with cDNA of hemocytes collected on day 0, 2, 6, 8 and 10 after infection with *N. bombycis* along with non-infected control. RT-PCR data on melanisation genes showed low level expression in the early days of infection. *Paralytic peptide* gene expression increased by 1.05 fold on day 6 and by 0.84 fold on day10 (Fig. 3A) followed by the increase of PP titer on day 10. Similarly *DDC* gene expressed low on day 6 and followed by enhanced (4.59 ± 0.065) level on day 10 after infection. Prophenol oxidase activating enzyme (*PPAE*) showed lower expression in early days of infection followed by enhanced expression by 1.14 fold on day 6 and then decreased (by -2.25 fold) on day 10 (Fig. 3A). In early days, low level of expression of pro- phenol oxidase (*PPO1)* is noted followed by down regulation by - 0.92 fold on day 6 and by - 3.8 fold, on day 10 after infection. *PPO2* enhanced its expression on day 6 by 0.24 fold while reduced by -2.46 fold on day 10.

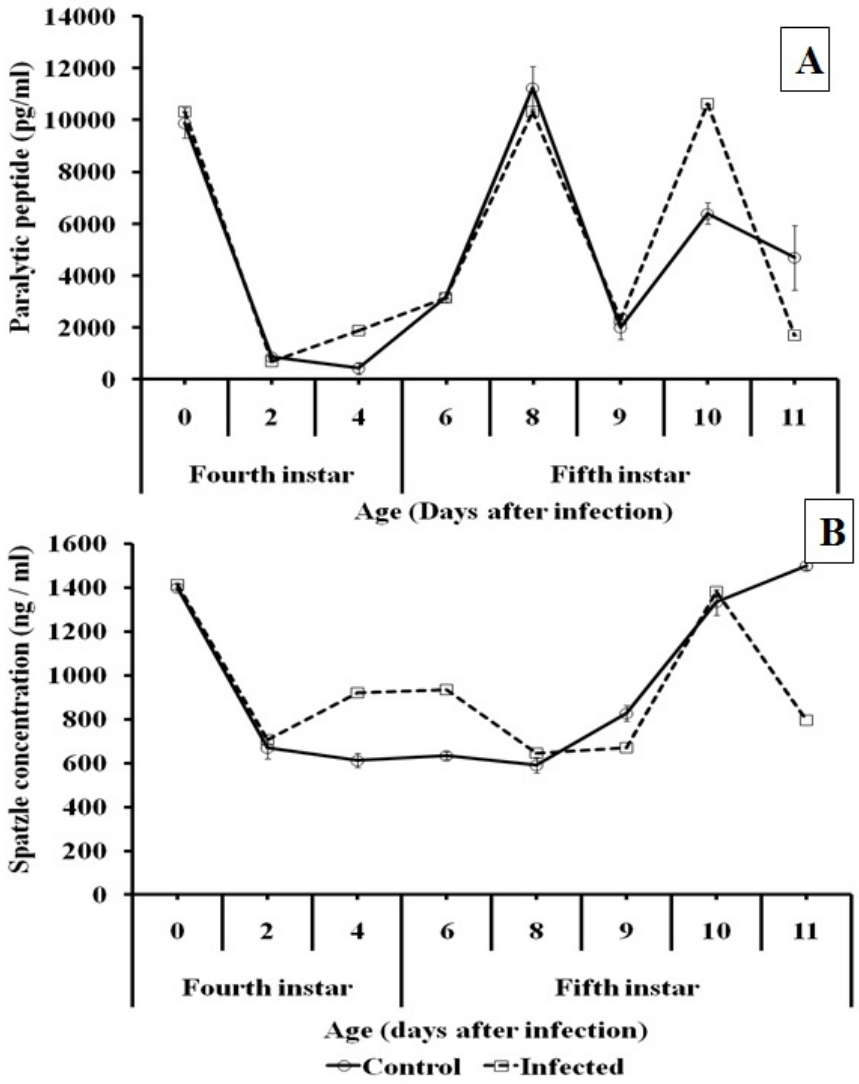


**Fig. 2. A-B:** Hemocyte cytokine titer: Paralytic peptide **(A)** titer showed increase on day 10 after *N. bombycis* infection and spatzle titer **(B)** increased on day 4 to 6 after the infection.

After *N. bombycis* infection in *B. mori*, PP titer is reduced in hemocytes leading to low melanisation supported by decreased gene expression of melanisation components. Melanization is a non-specific immune reaction. PO-dependent melanisation is the primary melanotic mechanism of silkworm (Lü, 2008). Moreover most of the

melanisation genes showed reduced expression level in the early stages of infection. Reduction in genic expression and low PP level revealed suppressed melanization in hemocytes. In midgut of *B. mori*, melanisation is decreased by *N. bombycis*- originated SERPIN showing suppression of immune activity in *B. mori* larva by parasitic action during the infection period (Ma *et al.*, 2013). In addition endoparasitoid wasps inhibit melanisation pathway by targeting serine protease, a key component of proPO cascade (Colinet *et al.*, 2013).

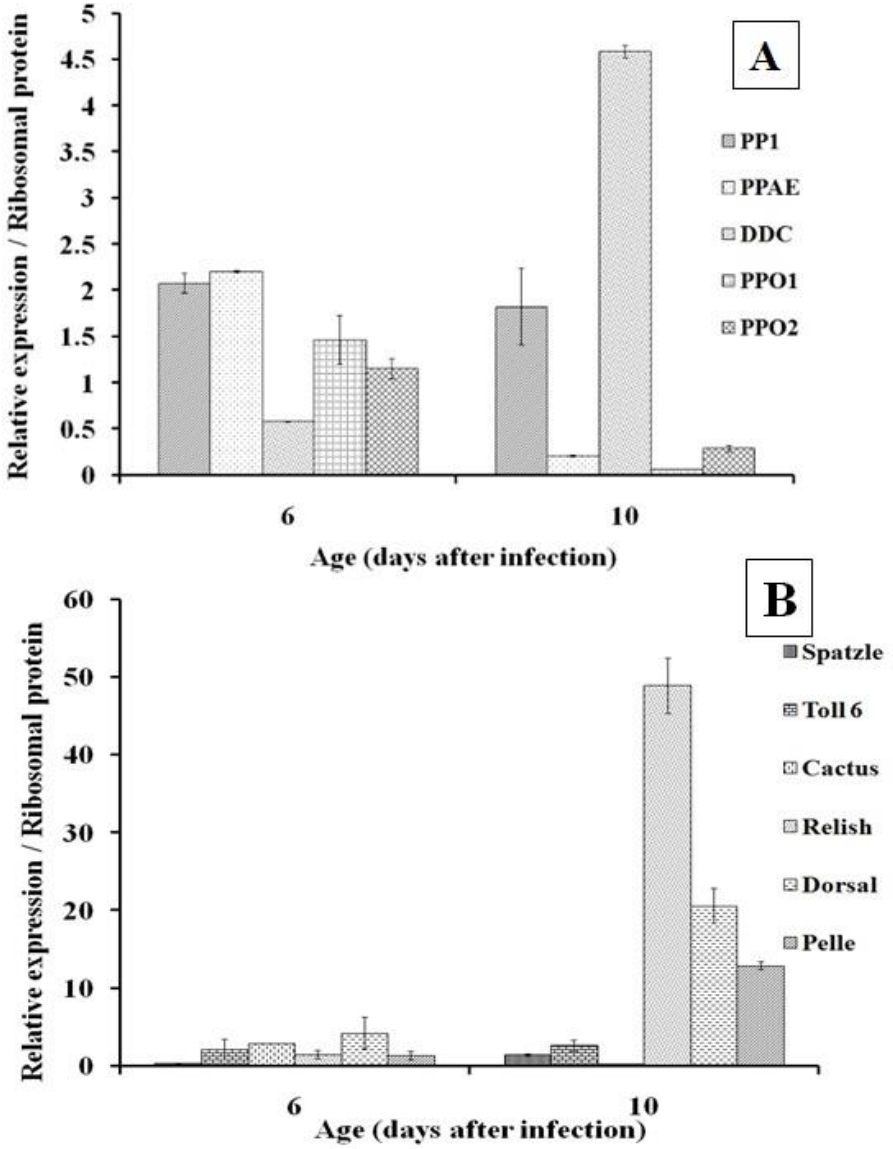


**Fig. 3. A-B:** Modulation in relative expression of melanisation component genes **(A)** and Toll pathway component genes **(B)** in hemocytes of *B. mori* larva after infection with *N. bombycis*.

**Innate immunity modulation**

In *B. mori*, infection by bacterial and fungal pathogens induce Toll pathway. It is suggested that different variants of Toll receptors are activated in *B. mori* (Ma *et al.,* 2013) however which toll variant regulate each specific pathogen is unclear. The Toll signalling pathway is capable of producing antimicrobial peptides to remove the effect of microorganisms (Yang et al, 2018). In Drosophila, toll pathway is activated by binding with the cytokine 'Spatzle' (Weber *et al.,* 2003). On receiving stimuli from fungal parasites, proteolytic cleavage of Spatzle is induced by serine protease. This cleavage initiates binding of C-terminal domain of Spatzle to surface of Toll receptor (Casanova-Torres and Blair, 2013). In *B. mori* hemocytes, spatzle titer increased significantly ($P < 0.009$) from day 4 to day 6 after infection, whereas on day 11 it decreased significantly ($P < 0.0006$) in comparison to control (Fig.

2B) showing requirement of the cytokine in early days of *N. bombycis* infection, possibly to elicit toll pathway.

Semi quantitative RT- PCR data showed differential expression of innate immune genes after infection however it did not show significant intensity increase showing reduced expression level. The Toll pathway components, *Toll, Spatzle, Relish* and *Pelle* did not show significant ($P < 0.42$) variation in intensity indicating low gene action. However qPCR showed low relative expression in mid infection followed by enhanced expression of *Relish, Dorsal* and *Pelle* in later stages, on day 10 (Fig. 3B).

Inactivation of Toll pathway genes early in the infection lead to low level of host defense reactions. RT-PCR showed lower spätzle amplicon density in the initial days of infection. On day 6 after infection *Spatzle* expression showed -1.63 fold reduction followed by 0.51 fold increase on day 10 showing cyclic *spatzle* gene activity (Fig. 3B). Spatzle titer did not vary upto day 4. Low gene expression coupled with low titer of spatzle revealed suppression of spatzle activity that caused lack of toll signalling as binding of spatzle with toll is a prime requirement to activate toll pathway (Weber *et al.,* 2003). Though Toll expression did not vary in initial stages of *N. bombycis* infection, expression enhanced by 0.89 fold on day 6 and 1.36 fold on day 10 (Fig. 3B). A simultaneous increase in spatzle (on day 10) is also noted indicating a positive feedback between *Spatzle* and *Toll* gene activation probably through multimerization and interaction (Hu *et al*., 2004; Parthier *et al*., 2014). Expression of *Pelle* enhanced by 0.187 fold on day 6 and by 3.64 fold on day 10. *Cactus* showed enhanced relative expression of 2.92 ± 0.02 on day 6, which then reduced to 0.215 ± 0.007 on day10. *Relish* upregulated on day 6 by 0.11 fold and by 5.60 fold on day 10 (Fig. 3B). Similarly relative expression of *Dorsal* on day 6 after infection was 4.25 ± 2.03 and it significantly enhanced to 20.6 ± 2.26 on day 10 (Fig. 3B). Lack of expression of toll pathway components in the initial stages of infection revealed suppression of toll-signalling as noticed earlier (Partheir *et al*., 2014) to distract the parasite from host responses. Recently it has been reported that Toll-1 and Toll-7 bind multiple Spatzle proteins and affect survival (Chowdhury *et al.,* 2019).

However increase in *Relish, Dorsal* and *Pelle* expression in later stages correlated with *Toll* expression indicated that accumulated effect of *N. bombycis* infection for 10 days elicited the host responses, only in later stages of infection.

## CONCLUSION

Under the suppressed innate immune responses like delayed Toll signalling and low melanization in the initial stages of *N. bombycis* infection, spore melanisation was repressed and the microsporidian could successfully establish itself in the host *B. mori* larva. Such molecular events could be used as targets to develop antibody – based early detection of microsporidian infection.

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