Journal of Invertebrate Pathology 109 (2012) 297–302

Contents lists available at SciVerse ScienceDirect

Journal of Invertebrate Pathology

journal homepage: www.elsevier.com/locate/jip

Survival and immune response of drones of a *Nosemosis* tolerant honey bee strain towards *N. ceranae* infections

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ARTICLE INFO

Article history:
Received 2 August 2011
Accepted 10 January 2012
Available online 20 January 2012

Keywords:
Nosema
Haploid
Microsporidian
Immune
Honey bee

ABSTRACT

Honey bee colonies (*Apis mellifera*) have been selected for low level of *Nosema* in Denmark over decades and *Nosema* is now rarely found in bee colonies from these breeding lines. We compared the immune response of a selected and an unselected honey bee lineage, taking advantage of the haploid males to study its potential impact on the tolerance toward *Nosema ceranae*, a novel introduced microsporidian pathogen. After artificial infections of the *N. ceranae* spores, the lineage selected for *Nosema* tolerance showed a higher *N. ceranae* spore load, a lower mortality and an up-regulated immune response. The differences in the response of the innate immune system between the selected and unselected lineage were strongest at day six post infection. In particular genes of the *Toll* pathway were up-regulated in the selected strain, probably is the main immune pathway involved in *N. ceranae* infection response. After decades of selective breeding for *Nosema* tolerance in the Danish strain, it appears these bees are tolerant to *N. ceranae* infections.

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1. Introduction

The honey bee, *Apis mellifera* can be infected by two microsporidian parasites, *Nosema apis* and *Nosema ceranae*. *N. apis* is an evolutionarily old pathogen of *A. mellifera* with moderate virulence and colonies can cure themselves under favorable environmental conditions (Zander, 1909; Chen et al., 2009). This relatively low virulence of *N. apis* is different from that of *N. ceranae*, a microsporidium originally found in the Asian honey bee *Apis ceranae* (Fries et al., 1996) which has been introduced into European honey bee populations (Higes et al., 2006; Fries et al., 2006) and is now widespread in *A. mellifera* populations across the globe (Chauzat et al., 2007; Cox-Foster et al., 2007; Klee et al., 2007). *N. ceranae* had been reported to have high virulence in both the colony and individual, but there are repeated reports suggesting the species is less virulent than *N. apis* (Paxton et al., 2007; Higes et al., 2008, 2009; Invernizzi et al., 2009; Gisder et al., 2010; Forsgren and Fries, 2010; Fries, 2010).

The *Nosema* infection cycle is well understood. Spores are ingested by the honey bee and germinate quickly in the midgut. The spores extrude the polar tube which penetrates the epithelial cells to release the sporoplasm directly into the cytoplasm (Higes et al., 2007; Fries, 2010). Within a week, the host epithelial cells are filled with offspring spores (Graaf et al., 1994; Gisder et al., 2011) and the cells burst to release a new generation of primary spores. If the honey bees can block the further infection, the primary spores develop into environmental spores and excreted through the feces. Otherwise, primary spores will germinate again and keep infecting more cells. However, the mechanisms of blocking *Nosema* infection in honey bees are still unclear.

In Denmark, bee keepers have been selecting colonies that have low *Nosema* infection level for decades (Traynor, 2008) and today *Nosema* is rarely found in bee colonies from these breeding lines. All four immune pathways – the Toll-pathway, the IMD-pathway, then JNK-pathway and the JAK/STAT-pathway have been identified in the *A. mellifera* genome (Evans et al., 2006) and respond to *Nosema* infection (Antúnez et al., 2009). Since the immune system has been shown to be involved in the *N. ceranae* infection responses (Antúnez et al., 2009) and the Danish breeding line has been selected for *Nosema* tolerance, we here screen how the immune system of the *Nosema* tolerance selected strain negotiates with this novel pathogen.

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doi:10.1016/j.jip.2012.01.004

2. Materials and methods

2.1. Bees

The same age sister virgin queens of the selected strain (*A. mellifera*) were provided by the Department of Integrated Pest Management Research Centre Flakkebjerg, Denmark. *A. mellifera carnica* queens from Germany that had not been selected for *Nosema* tolerance were kept at the apiary of the Martin-Luther-University Halle-Wittenberg served as controls. All queens were introduced into small colonies composed of about 2000 newly emerged workers. The queens were treated with CO_2 to initiate ovary activation (Mackensen, 1947) without mating so that they exclusively produced haploid drone offspring (Mackensen, 1951). We decided to screen the immune response of haploid drones rather than that of diploid workers because of their simple hemizygous genetic design which is free of allelic dominance interactions that might complicate any gene expression patterns of diploids. Frames of sealed drone brood and the worker brood were kept in an incubator (34 ± 1 °C). Freshly emerged drones of the selected queen and unselected queens were collected daily from the brood frames to provide *Nosema* free, age standardized individuals (0–24 h) for the artificial infection experiments. Freshly emerged workers of unselected queens in the apiary were served as nurse bees.

2.2. Infection and sampling

Fresh *N. ceranae* infected workers were provided by Laboratoire de Biologie et Protection de l'abeille, INRA Avignon, France. The abdomens of infected workers were homogenized in distilled water, filtered through filtering paper and centrifuged at 3220G for 10 min. The pellet was re-dissolved and centrifuged at 8700G for 5 min to purify the *N. ceranae* spores. Spores were counted using a Fuchs-Rosenthal haemocytometer and the *Nosema* species was verified by a standard PCR protocol (Hamiduzzaman et al., 2010).

Drones were individually fed with 2 μl sucrose solution containing $\sim 10^5$ *N. ceranae* spores. Drones that did not consume the entire volume were discarded. For each experimental group, 45–50 drones and 20 uninfected nurse workers were housed in a wooden cage (depth 13 cm × width 10 cm × height 11.5 cm) at 34 ± 1 °C, 60% rel. hum. Three cage replicates were conducted for selected strain, unselected strain and control group, respectively. Drones of the unselected strain received 2 μl sucrose solutions without any *Nosema* spores served as the control group.

Three drones were sampled from each cage every 24 h. Dead bees were removed and recorded until day eight post infection. The ventriculi of all sampled drones were removed and homogenized in 500 μl distilled H_2O individually. 10 μl Of this solution was used to count *N. ceranae* spores in a Fuchs-Rosenthal haemocytometer.

2.3. cDNA synthesis and quantative real-time PCR (qPCR)

After the gut removal for *N. ceranae* spore counting, the abdomen was immediately preserved in RNA later (Sigma–Aldrich) and stored at −80 °C until RNA was extracted (RNeasy Mini kit, Qiagen). RNA concentration and quality (absorption ratio 260 nm/280 nm) were spectrophotometrically measured (Nanodrop 1000, Pequlab). Equal amounts of RNA extracts of three drones of the same strain and same sampling date were pooled.cDNA was synthesized by mixing 80 ng of the RNA pools of three drones with 0.8 μl (0.5 μg/μl) oligo-dT18 primer (Fermentas, St. Leon-Rot, Germany) in 10 μl RNAse free water and incubated 5 min at 70 °C. Next 3.4 μl 5 × RT buffer (Promega, Mannheim, Germany), 0.8 μl dNTPs(10 mM), 0.4 μl M-MLV Revertase (200 u/μl, Promega, Mannheim, Germany), 0.2 μl Ribolock RNAse inhibitor (40 u/μl, Fermentas, St. Leon-Rot, Germany), 1.6DTT(0.1 mM) were added and incubated at 42 °C for 2 h and extending 15 min at 70 °C. Finally, the synthesized cDNA was diluted 1:10 with DEPC-water (DNase & RNase free water) for subsequent qPCR reactions.

1 μl Of this diluted cDNA was added to 5 μl SensiMix of the SYBR & Fluorescein kit (SYBR-Green, Bioline, Luckenwalde, Germany), 0.2 μl of the specific gene primer (10 mM) (Table 1) and 3.6 μl DEPC water for qPCR assay. After an initial phase of 95 °C for 3 min, the temperature cycle was as follows: 95 °C for 15 s; 60 °C for 30 s; 72 °C for 30 s for 40 cycles including plate read in every cycle. Finally melting curves were recorded by increasing the temperature from 50 °C to 90 °C.

Thirty three immune genes (Evans, 2006) covering all four immune pathways were amplified together with five reference genes (Yang and Cox-Foster, 2005; Van Hiel et al., 2009; Jarosch and Moritz, 2011) for all samples for each day from day one to six post infection. Immune genes and reference genes of each drone were run in the same 96 well plate to avoid plate variance. Each gene per sample was run in duplicate.

2.4. Statistical analysis

The temporal analysis of spore loads in the guts of drones of differences between the two honey bee strains were analyzed by Univariate Analysis of Variance, with strain and day post infection as fixed factor and replicates as a random factor using the SPSS 16.0 package. Survival of the two honey bee strains and the control group were analyzed with Kaplan–Meier procedure (SPSS 16.0).

The Opticon Monitor 3 (Bio-Rad, Munich, Germany) software was used to compute the C_t values and the PCR efficiency was calculated for individual reaction with the qpcR package (Spiess and Ritz, 2010), R (Hornik, 2011). The mean amplification efficiency of each gene over all samples were used for subsequent computations. Immune gene expression comparisons between two strains were conducted using the limma function of HTqPCR package (Dvinge and Bertone, 2009), R (Hornik, 2011). Genes that showed significant differences in expression levels between the two strains were further analyzed with an ANOVA to compare the relative expression differences among the two strains and the uninfected control group (SPSS 16.0).

The relative gene expression was computed as follows:

$$r = \frac{\sqrt[n]{\prod_{i=1}^{n} E_i^{C_t(i)}}}{E^{C_t(t\arg et)}}$$

where E is the PCR amplification efficiency; C_t is cycle threshold; i is ith reference gene; n is number of reference gene; r is the relative gene expression.

3. Results

3.1. Spore load dynamics and drone mortality

Spores were not found in the uninfected control group. During the first four days after the infections, the *N. ceranae* spore load in the infected drones' guts showed no significant differences between the two strains. However, from day five onward the number of spores in the drones of the selected strain was significantly higher than in the unselected strain ($p < 0.001$). This difference was largest on day six with more than an order of magnitude more spores in drones of the selected strain (8.2×10^5 vs 7×10^4, selcted vs unselected, $p < 0.001$) (Fig. 1). At day eight however the spore titers became more similar again. However, in spite of this much

Table 1
Tested immune genes, associated pathways and primers. Categories: P = pathogen or bacteria receptor; T = trans-membrane signal receptor; E = end product; K = kinase; TR = transcription regulator; R = reference gene.

Gene name	Pathway	Category	F. primer	B. primer
Abaecin	Toll	E	CAGCATTCGCATACGTACCA	GACCAGGAAACGTTGGAAAC
AmPPO	Toll	E	AGATGGCATGCATTTGTTGA	CCACGCTCGTCTTCTTTAGG
Apidaec	Toll	E	TAGTCGCGGTATTTGGGAAT	TTTCACGTGCTTCATATTCTTCA
Apisimin	Toll	E	TGAGCAAAATCGTTGCTGTC	AACGACATCCACGTTCGATT
Cactus-1	Toll	K	CACAAGATCTGGAGCAACGA	GCATTCTTGAAGGAGGAACG
Cactus-2	Toll	K	TTAGCAGGACAAACGGCTCT	CAGAAAGTGGTTCCGGTGTT
Defensin-2	Toll	E	GCAACTACCGCCTTTACGTC	GGGTAACGTGCGACGTTTTA
Defensin-1	Toll	E	TGCGCTGCTAACTGTCTCAG	AATGGCACTTAACCGAAACG
Dorsal-2	Toll	TR	TCACCATCAACGCCTAACAA	AACTAACACCACGCGCTTCT
Hymenopt	Toll	K	CTCTTCTGTGCCGTTGCATA	GCGTCTCCTGTCATTCCATT
Lys-1	Toll	E	GAACACACGGTTGGTCACTG	ATTTCCAACCATCGTTTTCG
Lys-2	Toll	E	CCAAATTAACAGCGCCAAGT	GCAATTCTTCACCCAACCAT
Lys3l	Toll	E	ATCTGTTTGCGGACCATTTC	TCGATGAATGCGAAGAAAATC
Myd88	Toll	K	TCACATCCAGATCCAACTGC	CAGCTGACGTTTGAGATTTTTG
PGRP9710	Toll	P	TTTGAAAATTTCCTATGAAAGCA	TTTTTAATTGGTGGAGATGGAAA
PGRPSC2505	Toll	P	TAATTCATCATTCGGCGACA	TGTTTGTCCCATCCTCTTCC
PGRPSC4300	Toll	P	GAGGCTGGTACGACATTGGT	TTATAACCAGGTGCGTGTGC
PPPOact	Toll	E	GTTTGGTCGACGGAAGAAAA	CCGTCGACTCGAAATCGTAT
Spaetzle	Toll	K	TGCACAAATTGTTTTTCCTGA	GTCGTCCATGAAATCGATCC
Toll	Toll	T	TAGAGTGGCGCATTGTCAAG	ATCGCAATTTGTCCCAAAAC
Dredd	Imd	K	GCGTCATAAAGAAAAAGGATCA	TTTCGGGTAATTGAGCAACG
Imd	Imd	T	TGTTAACGACCGATGCAAAA	CATCGCTCTTTTCGGATGTT
Kenny	Imd	K	GCTGAACCAGAAAGCCACTT	TGCAAGTGATGATTGTTGGA
Relish	Imd	TR	GCAGTGTTGAAGGAGCTGAA	CCAATTCTGAAAAGCGTCCA
Tak-1	Imd	K	ATGGATATGCTGCCAATGGT	TCGGATCGCATTCAACATAA
Demeless	JAK/STAT	P	TTGTGCTCCTGAAAATGCTG	AACCTCCAAATCGCTCTGTG
Hopscotch	JAK/STAT	K	ATTCATGGCATCGTGAACAA	CTGTGGTGGAGTTGTTGGTG
TepA	JAK/STAT	E	CAAGAAGAAACGTGCGTGAA	ATCGGGCAGTAAGGACATTG
Basket	JNK	K	AGGAGAACGTGGACATTTGG	AATCCGATGGAAACAGAACG
Dscam	JNK	E	TTCAGTTCACAGCCGAGATG	ATCAGTGTCCCGCTAACCTG
EGFlikeA	JNK	E	CATTTGCCAACCTGTTTGT	ATCCATTGGTGCAATTTGG
Hemipterous	JNK	K	CACCTGTTCAGGGTGGATCT	CCTTCGTGCAAAAGAAGGAG
ß-Actin		R	ATGCCAACACTGTCCTTTCTGG	GACCCACCAATCCATACGGA
RP49		R	CGCTACAAGAAGCTTAAGAGGTCAT	CCTACGGCGCACTCTGTTG
Gpdh56		R	GGATCAGGAAATTGGGGTTC	CGGAAGCTTATGTCCTGGAA
Gpdh-1		R	GCTGGTTTCATCGATGGTTT	ACGATTTCGACCACCGTAAC
EF		R	GATGCTCCAGGCCACAGAGA	TGCACAGTCGGCCTGTGAT

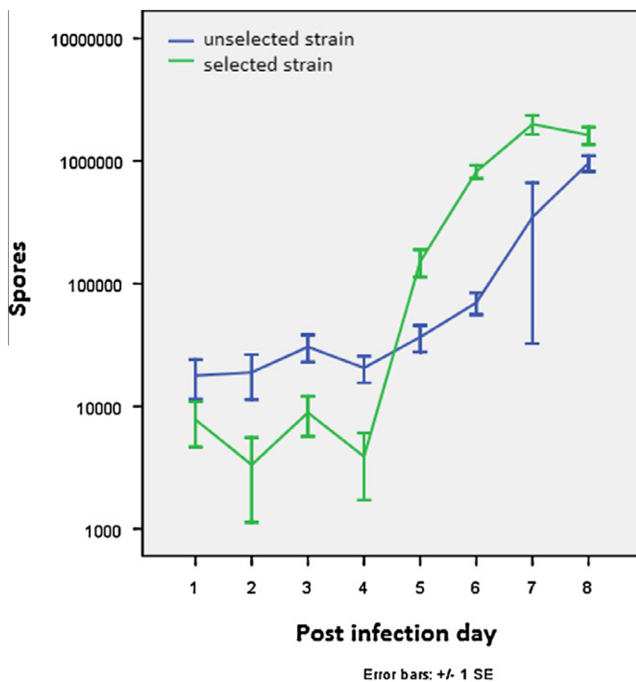

Fig. 1. Increase in *N. ceranae* spore load in the gut of artificially infected drone of the selected and unselected strain. Both strains were fed with the same number of *N. ceranae* spores. From 5 days post infection, the spore multiplication in selected tolerant strain was much higher than in the unselected strain.

higher spore load, the drones of the selected strain had a significantly lower mortality than those of the unselected strain (Log Rank, $p < 0.001$). Moreover the mortality of the selected drones was not significantly different from the uninfected control group (Log Rank, $p = 0.25$) (Fig. 2). The mortality in the unselected strain was significantly higher than the control group ($p < 0.001$). More than 90% of the drones of the selected strain were still alive even on day eight post infection whereas 40% the drones of unselected strain were dead. The highest mortality occurred on day two post infection in drones of the unselected strain (Fig. 2) (Table S1).

3.2. Reference and immune gene expression

Three genes from the five chosen reference genes (ß-Actin, RP49 and GAPDH56) were significantly differentially expressed between drones of selected and unselected population ($p < 0.05$) and hence unsuited and excluded as a reference in the further analyzes. The two remaining reference genes GAPDH-1 and EF showed no co-regulation with the treatment and were used for the normalization of the immune gene expression levels.

All 33 genes tested, showed no significant differences in expression levels between the selected strain and unselected strain during the first five days after the infection. However, on day six post infection, six immune genes from three pathways showed significantly differential regulation between the two strains (adjusted $p < 0.05$) (Fig. 3). The expression levels of these six genes were therefore further compared with the control groups to identify which strain deviated from the uninfected drones.

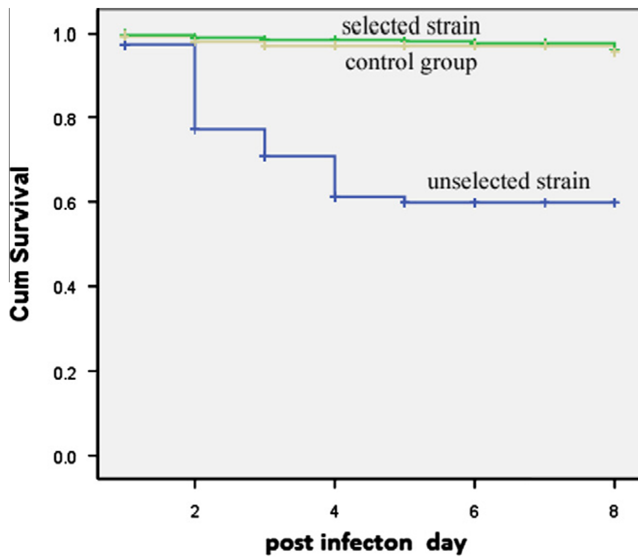

Fig. 2. Time series mortality. Three drones that were sampled every day from each cage. These three drones were treated as sensored in the survival analysis. Drones in the unselected strain had significantly higher mortality (Kaplan–Meier procedure) whereas there was no difference in survival between the uninfected control group and the drones from the selected lineage.

3.3. Toll pathway

All screened Toll pathway genes were up-regulated after the infection in both the selected and the unselected strain. The peptidoglycan recognition protein-SA (PGRPSC4300, GB15371) was significantly up regulated on day one post infection compared to the control group ($p < 0.001$). This up-regulation decreased steadily over the subsequent days in both strains until this gene was close to the expression level of the uninfected control group in the selected strain day six post infection, and even significantly down regulated in the unselected strain ($p < 0.05$). Toll (GB18520), lysozyme-2 (lys-2, GB15106) and dorsal-2 (GB18032) showed a similar but less pronounced expression dynamics and only on day six was there a significant difference in the expression levels between the selected and the unselected strain ($p < 0.05$) (Fig. 3).

3.4. IMD pathway and JAK/STAT pathway

All screened genes of these two pathways (IMD and JAK/STAT) were up-regulated in the infected drones of both strains during the first five days after the infection. Again significant differences only occurred on day six post infection when Dredd (GB17683, Imd pathway) up regulated in the selected strain ($p < 0.01$) but down regulated in the unselected strain. The gene domeless (GB16422) from the JAK/STAT pathway was similarly up-regulated after the infection in both strains ($p < 0.01$), but again significantly down-regulated in the unselected strain on day six post infection (Fig. 3).

4. Discussion

As shown before for workers (Antúnez et al., 2009), also in our experiments with drones immune genes were involved in the response to *N. ceranae* infection, resulting in a general increase in immune activity in response to the infection. The gene expression levels from day one to five post infection in both the selected and the unselected strain were higher than in the uninfected controls. However there were no significant differences between the gene expression levels of drones from the selected and the unselected strain. Only on day six post infection could we observe reduced expression levels in genes of the innate immune system in the unselected strain, similar to the depression of the immune system in workers (Antúnez et al., 2009).

Although *Nosema* was similar with the fungi (Liu et al., 2006) and is not a bacterial pathogen, the strongest response towards the infection was that of the bacterial recognition receptor PGRPSC4300 of the Toll pathway. The membrane-spanning Toll receptor known to initiate the cell immune cascade (Imler et al., 2004) was also up-regulated as was Dorsal which is involved in melanization and antimicrobial effectors production. After day four post infection, the overall immune activity decreased to reach expression levels close to uninfected control group (for the selected strain) or even significantly less (for the unselected strain) on day six post infection. In spite of the receptor activation, the infection did apparently not further enhance the subsequent immune gene cascade of the drones at this stage. In addition the trans-membrane receptor domeless in JAK/STAT was also up regulated immediately at the beginning of the infection. But we did not find any expression differences neither on hopscotch nor thioester containing protein between the selected and unselected strain. Since Dredd was the only gene from the IMD pathway showed a different expression on day six post infection between the selected and unselected strain. There is no clear cascade pattern how IMD pathway and/or the JAK/STAT pathway initiate the response to the *N. ceranae* infection, or were secondarily activated by other triggers. Alternatively, the immune genes in IMD pathway and the JAK/STAT were regulated at post expression level. Therefore, we cannot find the significant difference on the gene expression.

Higes et al. (2007) shows that the number of infected epithelial gut cells markedly increase on day six post infection and start degeneration after day seven post infection. Also in our study *Nosema* spores titers rapidly increased after four days post infection in both strains. The *N. ceranae* spore titer was even larger in drones of the selected strain in conjunction with lower mortality than those of the unselected strain. The high mortality in the first few days after infection is not due to the replication of *Nosema* which occurs at a later stage. But adding *N. ceranae* spores to the inoculation diet did significantly increase the mortality, because the control drones survived. This may be due to the gut damage during the initial infection, but it may also be due to the behavior of the host workers towards the infected drones. It is important to note that these highly sensitive drones died and could not establish high spore loads after *Nosema* replication. The variance in survival might result from the variance in the initial feeding of spore solutions. Although we tried to control the initial spore dose, variance among the actual numbers of spore administered to the drones is inevitable. We interpret the result such that only those susceptible drones which had received a low spore number survived, which in turn eventually resulted in the low spore load at the end of the experiment. But we have not found a significance difference on the spore load between the dead and alive drones, which might because the highest mortality was on day two post infection and most dead drones were before day four post infection. So the spore load was not significantly different at this early phase of the infection. We do not know the actual mechanisms how drones of selected strain fight against *N. ceranae* infection and how an initial *N. ceranae* infection can kill the drones of unselected strain. QTL mapping and a histological examination of the gut tissue will be suitable to test these hypothesis.

After decades of selective breeding for *Nosema* tolerance in the Danish honey bee strain, it appears these bees are not only tolerant to *N. apis* but at least the drones are also tolerant to *N. ceranae*. The selected strain showed a very low mortality and a high tolerance to *N. ceranae* spore load. If not kept in the cages, under natural conditions and at the apiary the drones would simply expel the high

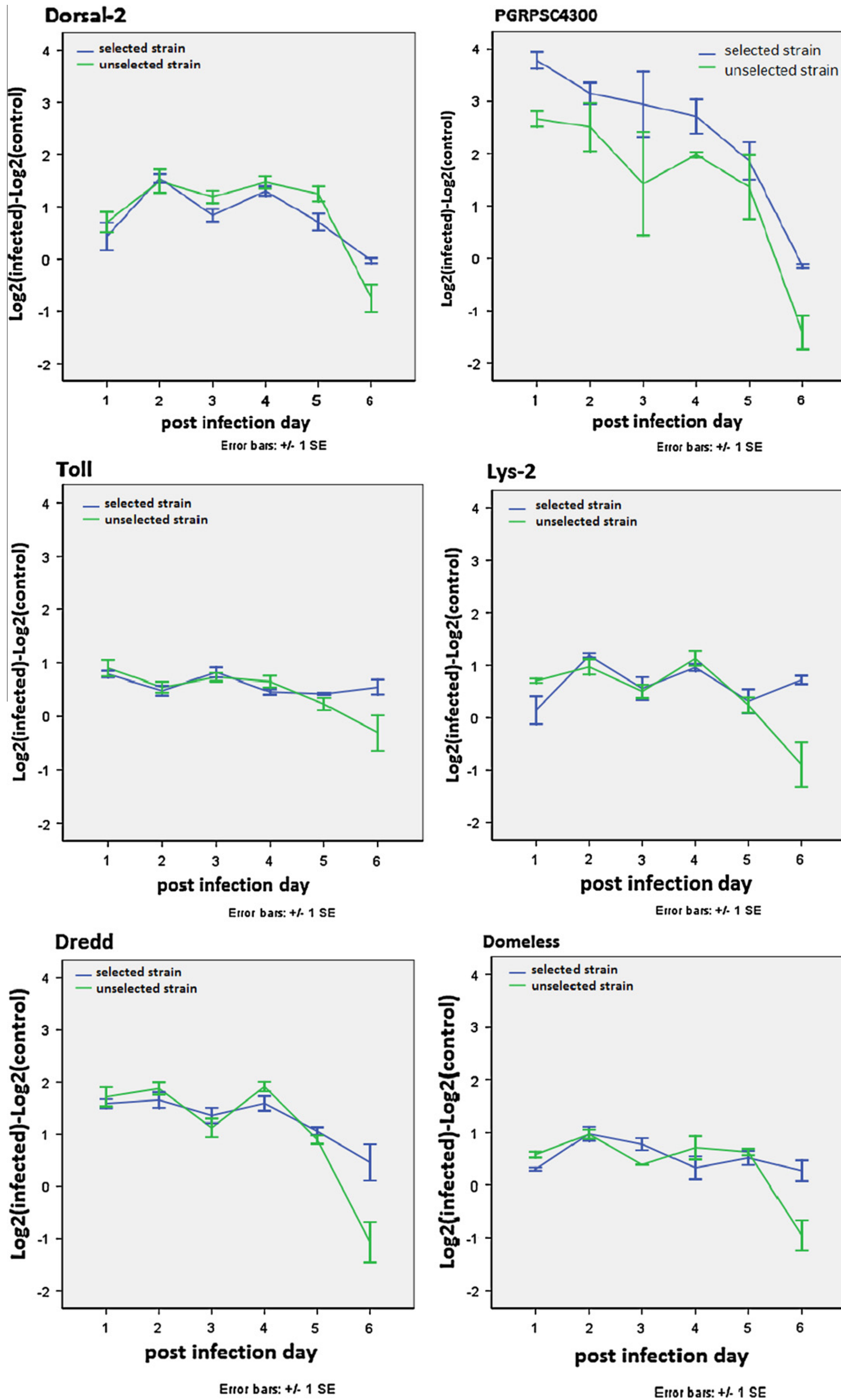

Fig. 3. Immune gene expression levels (log transformed) in relation to the uninfected controls and normalized for two housekeeping genes.

spore load outside the colony during defecation flights. In spite of the strong correlation between survival and immune gene expression we cannot exclude that also genetic mechanisms other than the immune pathways drive the tolerance to *N. ceranae*. Toll pathway showed a consistent cascade responded to the *N. ceranae* infection. Bacterial receptor, trans-membrane signal receptor and transcription regulator of the antimicrobial were all significantly up-regulated in the selected strain. *N. ceranae* tolerant genes may strongly correlated with the immune response by directly enhance the immune gene expression and suppress the virulence of *N. ceranae*. Nevertheless the response of the innate immune system of the drones of the strain selected for *Nosema* tolerance turned out to be significantly stronger than for the unselected strain that suffered a high mortality.

Acknowledgments

We thanks M. Lattorff and B. Fouks for discussion and revising the manuscript. This work was supported by the European Union's Seventh Framework Programme (FP7/2007-2013 and FP7/2007-2010) under grant agreement BEE DOC FP7-KBBE-2009-3 244956 CP-FP, Deutsche Forschungsgemeinschaft Project DFG SPP 1399 (MO 373/26-1) and China Scholarship Council.

Appendix A. Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jip.2012.01.004.

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