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# *Dicer* regulates *Nosema ceranae* proliferation in honeybees

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## Abstract

***Nosema ceranae*** **is a microsporidian parasite that infects the honeybee midgut epithelium. The protein-coding gene *Dicer* is lost in most microsporidian genomes but is present in *N. ceranae*. By feeding infected honeybees with small interfering RNA targeting the *N. ceranae* gene coding *Dicer* (siRNA-Dicer), we found that *N. ceranae* spore loads were significantly reduced. In addition, over 10% of total parasite protein-coding genes showed significantly divergent expression profiles after siRNA-Dicer treatment. Parasite genes for cell proliferation, ABC transporters and hexokinase were downregulated at 3 days postinfection, a key point in the middle of parasite replication cycles. In addition, genes involved in metabolic pathways of honeybees and *N. ceranae* showed significant co-expression. Furthermore, the siRNA-Dicer treatment partly reversed the expression patterns of honeybee genes. The honeybee gene *mucin-2-like* showed significantly upregulation in the siRNA-Dicer group compared with the infection group continually at 4, 5 and 6 days postinfection, suggesting that the siRNA-Dicer feeding promoted the strength of the mucus barrier resulted from interrupted parasite proliferation. As the gene *Dicer* broadly regulates *N. ceranae* proliferation and honeybee metabolism, our data suggest the RNA interference pathway is an important infection strategy for *N. ceranae*.**

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## Introduction

The microsporidia comprise a large group of spore-forming unicellular fungal parasites (Thomarat *et al*., 2004; Lee *et al*., 2017). All known microsporidia parasite species exclusively infect animal hosts, and most infect invertebrates (Didier, 2005; Lom and Dykova, 2005; Cornman *et al*., 2009; Cuomo *et al*., 2012; Chen *et al*., 2013; Pan *et al*., 2013). Parasite virulence and the responses of hosts towards infections are different depending on the parasite species. Microsporidian genomes are generally compact (2–10 Mbp), except *Edhazardia aedis* (51 Mbp, infecting the yellow fever mosquito) (Pombert *et al*., 2012). As an intracellular parasite, the microsporidia cannot reproduce outside host cells. Although classic mitochondria are absent, microsporidia carry tiny mitochondrially derived organelles called mitosomes, suggesting that parasites fuel their rapid proliferation with energy from their hosts (Burri *et al*., 2006). It is then not surprising that infection changes host metabolic dependency. Hexokinase, which catalyses the conversion of glucose for the glycolytic and pentose phosphate pathways, is reported to be a virulent factor for the microsporidia parasite *Nematocida* (Cuomo *et al*., 2012). The gene coding hexokinase has acquired a secretion signal sequence that allows this enzyme into host cells to increase the production of amino acids, lipids and nucleotides (Robey and Hay, 2006). Microsporidia are also reported to inhibit the apoptosis of infected host cells to maximize reproduction. Different host genes of the apoptosis pathway were reported to be regulated to inhibit the apoptosis across different parasite species (del Aguila *et al*., 2006; Higes *et al*., 2013; He *et al*., 2015; Doublet *et al*., 2017; Martín-Hernández *et al*., 2017). Additional studies have tried to identify the important proteins for microsporidian infection, including parasite polar tube proteins, cell wall proteins, adenosine triphosphate

transporters and host cuticle proteins (Xu and Weiss, 2005; Paldi *et al*., 2010; Li *et al*., 2016, 2012 ; Han *et al*., 2017). Nevertheless, there have been few attempts to identify more broadly the pathways used by microsporidia to infect hosts, and the counter-defences hosts might use to reduce the impacts of infection.

*Nosema ceranae* is a microsporidian parasite of honeybees (Fries *et al*., 1996). After ingestion, spores germinate in the midgut and inject the sporoplasm into host cells through extruded polar filaments. Cell proliferation starts from meronts, leading to sporonts and finally forming mature spores (Higes *et al*., 2007; Gisder *et al*., 2011). Infection shows profound effects on honeybee metabolism, flying behaviour, pheromone profiles and life span (Antúnez *et al*., 2009; Mayack and Naug, 2009; Martín-Hernández *et al*., 2011; Dussaubat *et al*., 2013; Goblirsch *et al*., 2013). Two virulence factors have been reported in *N. ceranae*. First, *N. ceranae* is reported to inhibit the apoptosis of the infected honeybee cells, which maximizes spore proliferation (Higes *et al*., 2013). *N. ceranae* also has an annotated hexokinase coding gene with a secretion sequence, which regulates honey metabolism to fuel spore proliferation (Cornman *et al*., 2009). We recently found the gene *Dicer* is an important regulator for *N. ceranae* proliferation (Huang, *et al*., 2016a). In order to understand the mechanism of *N. ceranae* proliferation regulation by *Dicer*, we treated honeybees infected with *N. ceranae* with small interfering RNA (siRNA) targeting the parasite gene for *Dicer*. We quantified gene expression at the transcriptomic level for both honeybee and the parasite across a time series covering a complete parasite proliferation cycle. We aimed to (1) quantify the effects of *Dicer* on parasite spore proliferation, (2) quantify the effects of *Dicer* on host and parasite gene expression, and (3) identify any co-expressed genes between the host and parasite during the infection.

## Results

### *Transcriptome profile of the honeybees and putative gene functions*

*N. ceranae* spores were not found in the uninfected group. On average, the percentage of reads aligned to the honeybee genome decreased from 80.5% (infection group), 80% (siRNA-scramble group) and 80% (siRNA-Dicer group) at 1 day postinfection (dpi) to 55% (infection group), 55.5% (siRNA-scramble group) and 60.5% (siRNA-Dicer group) respectively at 6 dpi (Table 1). The postinfection day [df = 5, $P < 0.001$, analysis of variance (ANOVA)] and treatment (df = 2, $P < 0.05$, ANOVA) showed significant effects on the number of reads that could be aligned to the honeybee genome, as expected from an increase in the amount of parasite RNA.

In order to normalize the foreign siRNA effect, the significantly regulated genes between the siRNA-scramble group and the infection group were removed for further analysis. There were 29, 56, 28, 30, 28 and 26 genes significantly regulated from 1 to 6 dpi, due to the feeding of siRNA-Dicer (Fig. 1; file S1). Significantly regulated genes were not evenly distributed over 6 dpi ($P < 0.01$, df = 5, Pearson's $\chi^2$ test), suggesting the effects of

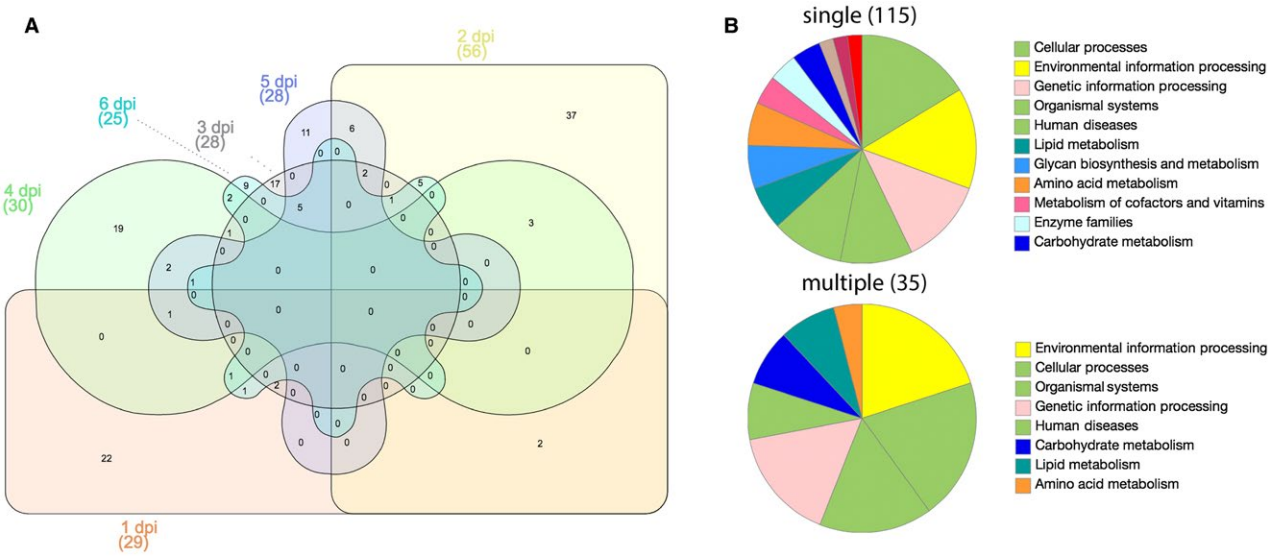


**Figure 1.** (A) Six-way Venn diagram showing the distribution of shared significantly regulated honeybee genes due to small interfering RNA (siRNA)-Dicer feeding over 6 days postinfection (dpi). Numbers of clusters are provided in the interactions. The total number of significantly differentially regulated host genes are provided for each dpi. (B) The functional category of significantly regulated genes due to siRNA-Dicer feeding at one time point (115 genes) and multiple time points (35 genes). The corresponding colour is shown on the right according to KEGG pathway database. [Colour figure can be viewed at wileyonlinelibrary.com]

**Table 1.** Percentage of reads mapped to host and parasite genomes and the number of significantly regulated genes from 1 to 6 days postinfection (dpi)

| | Reads mapped to genome at each sampling point, dpi (%) | | | | | | No. of genes differentially expressed at each sampling point, dpi | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Treatment group | 1 | 2 | 3 | 4 | 5 | 6 | 1 | 2 | 3 | 4 | 5 | 6 |
| **Parasite** | | | | | | | | | | | | |
| Infection | 0 | 0 | 0.4 | 0.5 | 1.2 | 3.5 | 0 | 14 | 284 | 109 | 0 | 0 |
| siRNA-Dicer | 0 | 0 | 0 | 0.3 | 2.3 | 4.8 | | | | | | |
| siRNA-scramble | 0 | 0 | 0.4 | 0.3 | 1.9 | 4.1 | | | | | | |
| **Host** | | | | | | | | | | | | |
| Infection | 80.5 | 72.3 | 63 | 64.7 | 60.3 | 55.0 | 29 | 56 | 28 | 30 | 28 | 26 |
| siRNA-Dicer | 80 | 71.6 | 70 | 64.9 | 58.5 | 60.5 | | | | | | |
| siRNA-scramble | 80 | 74.5 | 67.1 | 65.7 | 62.4 | 55.5 | | | | | | |

siRNA-Dicer feeding is time specific. Overall, 150 honeybee genes were significantly regulated. Out of those 150 genes, 24 genes were significantly regulated at two time points and 11 genes were significantly regulated at three time points (Fig. 1). The reoccurrence of significantly regulated genes deviated from random over six time points ($P < 0.001$, Fisher's exact test), suggesting that siRNA-Dicer feeding causes the observed gene regulation instead of random effects.

In order to test whether siRNA-Dicer feeding reversed expression of honeybee genes regulated by *N. ceranae* infection, we compared an earlier messenger RNA (mRNA) sequencing dataset with this study (Huang, *et al.*, 2016b). The two studies used the same infection procedure and the same honeybee apiary. Out of identified 150 significantly regulated genes in the current study, 52 genes were found significantly regulated due to *N. ceranae* infection in the previous study. For those 52 genes, 16 genes were found with siRNA-Dicer-mediated expression patterns at the same postinfection day, ie while the gene is upregulated in the infection group compared with the uninfected group, but the same gene is then downregulated in the siRNA-Dicer group compared with the infection group at the same time points spanning 6 dpi. The pattern significantly deviates from random ($P < 0.01$, df = 1, Pearson's $\chi^2$ test). This indicates that parasite siRNA-Dicer feeding partly reverses the disease response patterns of honeybee genes.

The functions of significantly regulated honeybee genes included apoptosis (LOC552766), innate immune response (LOC724187, LOC727161), pathogen growth inhibitors (LOC410509), venom toxin (LOC100579018, LOC406141), glycolysis (LOC410005, LOC408603, LOC410743, LOC552425, LOC727333) and insect cuticle proteins (LOC724694, 724897). The gene *mucin-2-like* (LOC410509) was consistently significantly overexpressed in the siRNA-Dicer group compared with the infection group from 3 to 6 dpi. The gene cytochrome P450 (LOC409469) was also significantly overexpressed in the siRNA-Dicer group compared with the infection group at 1 dpi. By mapping the protein sequences of the significantly regulated genes to the KEGG pathway database, two pathway modules (nucleotide and amino acid metabolism) and one functional set (Wnt signalling) were identified. Additionally, 20 KEGG orthologue groups were found, covering metabolism, cell growth and death, longevity and infection responses (Fig. 1).

### *N. ceranae spore production and transcriptome profiling*

On average, the percentage of reads aligned to the *N. ceranae* genome increased from zero in three treatment groups at 1 dpi to 3.5% (infection group), 4.1% (siRNA-scramble group) and 4.8% (siRNA-Dicer group) at 6 dpi (Table 1). The postinfection day (df = 5, $P < 0.001$, ANOVA) had a significant effect on the number of reads aligned to the parasite genome. The increased number of aligned reads over six time points confirmed the infection success and the growth of the parasite. By counting the spore load of individual honeybees at 6 dpi, honeybees in the siRNA-Dicer group showed significantly fewer spores than the infection group and siRNA-scramble group ($P < 0.01$, Kruskal–Wallis ANOVA, Fig. 2). Even though the siRNA-scramble group showed high variance, the two replicates were not significantly different from each other (*t*-test, $P > 0.05$). Furthermore, gene expression across the two replicates was significantly correlated (Pearson's correlation, $P < 0.001$).

The expression of *Dicer* was downregulated in the siRNA-Dicer group compared with infection and siRNA-scramble groups at 3 dpi. But the difference was not statistically significant. Overall, the expression levels of 347 parasite genes were significantly regulated due to siRNA-Dicer feeding. In particular, 14, 284 and 109 genes were significantly regulated at 2 dpi, 3 dpi and 4 dpi respectively. Significantly regulated genes were not found at 1, 5 and 6 dpi. The number of significantly regulated genes was not evenly distributed over the infection period ($P < 0.001$, df = 5, Pearson's $\chi^2$ test). Among 347 significantly regulated genes, four genes were regulated at three time points, 52 genes were regulated at two time points and the remaining genes were regulated at a single time point. The frequency of up and downregulation is significantly deviated from random from 2–4 dpi ($P < 0.001$, df = 2, Pearson's $\chi^2$ test). Significantly

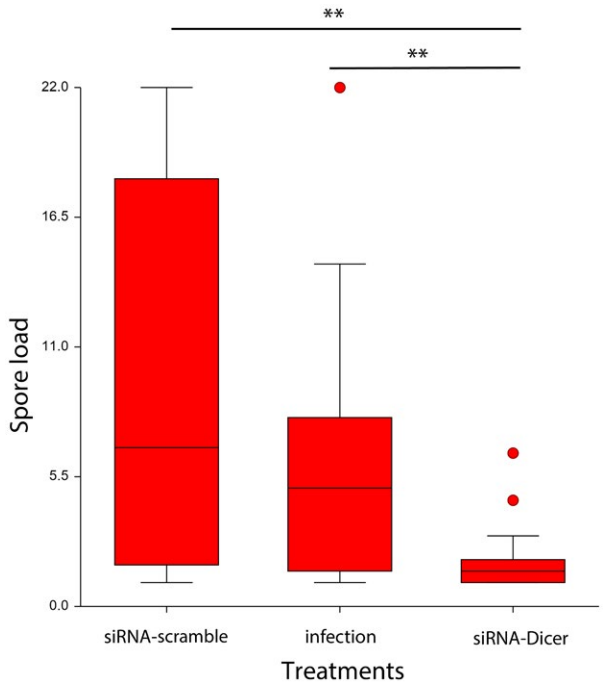


**Figure 2.** Box plot of *Nosema ceranae* spore loads in three treatment groups. All the honeybees were sampled to count the spore load after 6 days postinfection. Each individual honeybee midgut tissue was homogenized in distilled water (1 : 5000 dilution) to count the spore loads using a Fuchs–Rosenthal haemocytometer. Each homogenized solution was counted two times and the average of the two was used. Overall, the spore loads were statistically significantly different among small interfering RNA (siRNA)-Dicer group, siRNA-scramble group and infection group ($P < 0.01$, Kruskal–Wallis one-way analysis of variance). The honeybees of the siRNA-Dicer group showed significantly less spores than infection group and siRNA-scramble group ($P < 0.01$, Dunn's test). Even though the siRNA-scramble group showed high variance, the two replicates were not significantly different (*t*-test, $P > 0.05$). Statistically significant difference at <0.05 (*) and <0.01 (**). [Colour figure can be viewed at wileyonlinelibrary.com]

differentially expressed genes showed a strong tendency to be upregulated at 2 and 4 dpi and downregulated at 3 dpi ($P < 0.05$, $Z$ test). As an extreme case, all 14 significantly regulated genes were overexpressed in the siRNA-Dicer group at 2 dpi. Those 14 genes include five not observed at all in the infection group but highly expressed [>1500 counts per million reads (cpm)] in siRNA-Dicer (two proteasome AAJ76_6000110666 and AAJ76_1600058198; protein disulphide isomerase AAJ76_6000110666; septin like protein AAJ76_1000165053; enolase AAJ76_4300015194), four spore-wall-related genes (AAJ76_2000141845, AAJ76_2000146453, AAJ76_1600051435 and AAJ76_1200044876), one histone (AAJ76_1300019985) and one septin (AAJ76_1000142585). Three genes that were significantly regulated at three time points (AAJ76_2000146453, AAJ76_1600051435 and AAJ76_1200044876) were involved in spore wall formation (Fig. 3). The genes that were significantly regulated at two time points were also found involved in glycolysis (AAJ76_5600016640, AAJ76_1200023091), polar tube formation (AAJ76_1000109660, AAJ76_1900025375) and endocytosis (AAJ76_200030996). By aligning the protein sequences of significantly regulated genes to the KEGG pathway database, 45 KEGG pathway modules and 92 KEGG orthologue groups were identified, which include metabolism, transcription, translation, DNA repair and degradation, cell growth and death. In particular, two functional sets of transfer RNA biosynthesis and cell cycle transition (G1–S transition and G2–M transition) were identified (file S2).

### *Time series gene expression patterns of N. ceranae*

In order to understand the impacts of siRNA-Dicer feeding on *N. ceranae* gene expression across the entire experimental period, a time-series analysis of gene expression was performed. The expression patterns of 211 genes differed in the siRNA-Dicer group compared with the infection group, clustering into four groups (Fig. 4). All of those 211 genes were significantly differentially expressed from the mentioned transcriptome analyses. Those 211 genes showed a significant tendency to be regulated at multiple time points compared with the remaining 136 genes ($P < 0.05$, df = 1, Pearson's $\chi^2$ test). Also, among the four clusters, the number of time points when the genes were significantly regulated was significantly deviated from random ($P < 0.001$, df = 3, Pearson's $\chi^2$ test). For cluster 1, the variation is mainly at 3 dpi, when the genes were not expressed in the siRNA-Dicer group but highly expressed in the infection group. Out of 125 genes, 106 genes were significantly downregulated in the siRNA-Dicer group compared with the infection group from cluster 1. Additionally, 110 genes were significantly differentially expressed at 3 dpi. All of those 110 genes were downregulated or not expressed in the siRNA-Dicer group compared with the infection group at 3 dpi. The remaining 14 genes were downregulated or not expressed in the siRNA-Dicer group compared with the infection group at 4 dpi. This pattern suggested the genes had tended to be expressed at low level or not expressed in the siRNA-Dicer group at 3 dpi. For cluster 3, seven genes were differentially expressed at 2 dpi. All of those seven genes were overexpressed in the siRNA-Dicer group. There were 26 gene differentially expressed at 3 dpi, which were all downregulated in the siRNA-Dicer group. There were also 30 gene differentially expressed at 4 dpi, which were all overexpressed in the siRNA-Dicer group. For cluster 4, all eight genes were overexpressed in the siRNA-Dicer group at 3 dpi. Cluster 4 showed a reversed expression pattern (overexpression in siRNA-Dicer group) compared with cluster 1. For cluster 2 and cluster 3, the variation occurred at multiple time points.

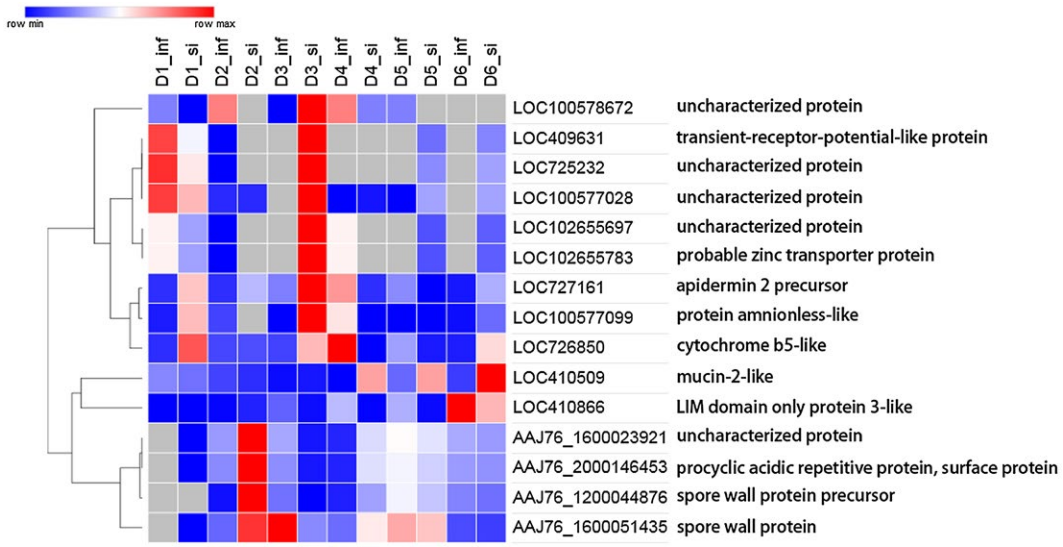


**Figure 3.** Heat map analysis of selected genes across all six days postinfection. Heat map of genes that were significantly regulated at three time points. There were 11 honeybee genes and four parasite genes. Two out of four parasite genes were associated with spore wall formation, which are essential for spore proliferation. Mucin and apidermin were also important for the honeybee defences. [Colour figure can be viewed at wileyonlinelibrary.com]

### *Gene interactions between host and parasite*

A significantly higher proportion of transcriptome was regulated in the parasite compared than in the host, resulting from siRNA-Dicer feeding ($P < 0.001$, df = 1, Fisher's exact test). The distributions of the significantly expressed genes among seven physiological categories (metabolism, environmental information processing, genetic information processing, cellular information processing, cellular processes, organismal systems, human diseases) were significantly different between the host and the parasite ($P < 0.001$, df = 5, $\chi^2$ test). By comparing the percentage of genes in each of the seven categories, metabolism and genetic information rank the first for the host and the parasite respectively, which partly manifests the responses towards the siRNA-Dicer treatment. By enrichment analysis, cell periphery, tissue development and ion binding were the most enriched for the parasite compared with nucleus, metabolism and heterocyclic compounds of the host. For the parasite, 325 genes were significantly co-expressed and clustered into two groups. Out of 150 host genes, 79 genes were significantly co-expressed and clustered into two groups. Reciprocal co-expression analysis was then performed, where the expression levels of parasite genes were used as a phenotype and this associated with groups of honeybee genes. Then the expression levels of honeybee genes were used a phenotype and this associated with parasite genes. The results showed that the expression levels of 25 honeybee genes were significantly associated with 138 *N. ceranae* genes (file S3). The distribution of associated genes in each cluster between the honeybees and *N. ceranae* were significantly deviated from random ($P < 0.05$).

## Discussion

Using RNA interference (RNAi) to suppress the expression levels of candidate genes is a powerful method for inferring gene function (Kanehisa and Goto, 2000; Scherr *et al*., 2003). When the candidate gene is a critical part of the RNAi pathway itself, such as *Dicer*, it can be extremely challenging to use siRNA to test for function (Niu *et al*., 2017). When the expression level of *Dicer* is too low, the efficiency of the RNAi pathway can be reduced (Niu *et al*., 2016). In our study, the expression level of the parasite gene *Dicer* was downregulated, but not statistically significant, after feeding the siRNA-Dicer at the sampling time points. This might because the expression level of *Dicer* was only changed within a short period of time, which was not captured at the sampling points. Nevertheless, if siRNA-Dicer has no significant effects on the gene expression level of *Dicer*, siRNA-Dicer should then act as a random foreign siRNA with similar effects to scramble or random siRNA. In contrast, siRNA-Dicer feeding did impact transcript abundances, by significantly increasing the number of reads aligned to honeybee and *N. ceranae* genomes in the siRNA-Dicer group compared with the infection group. As mature spores were protected by proteinaceous and chitin structured cell wall layers, TRIzol may not fully break the mature spore wall to extract mRNAs (Bohne *et al*., 2011). As a result, only mRNAs of the vegetative stage would be extracted, which might explain why higher proportions of reads were aligned in the siRNA-Dicer group than in the infection group. The impact of this on the transcriptomic analysis is not substantial because most of the mRNAs are dormant. Our aim is to quantify mRNAs during the

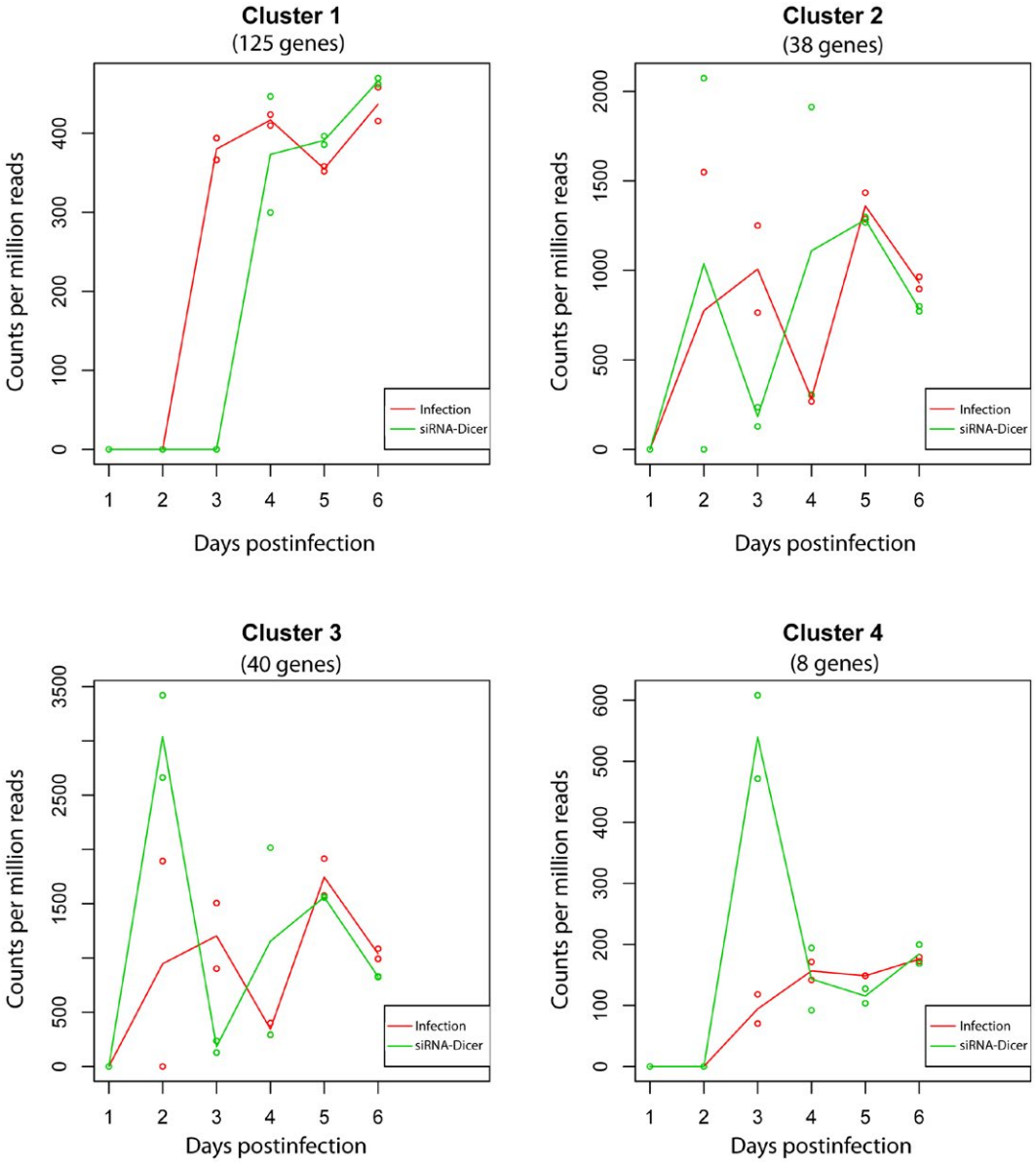


**Figure 4.** *Nosema ceranae* gene expression patterns during 6 days postinfection period. In total, the expression patterns of 211 genes were significantly regulated in the small interfering RNA (siRNA)-Dicer group compared with the infection group across the 6-day infection period, which were clustered into four groups. The *y*-axis is the median normalized counts per million reads. [Colour figure can be viewed at wileyonlinelibrary.com]

proliferation. The number of significantly regulated honeybee genes between the siRNA-Dicer group and the infection group after this correction was significantly deviated from random. Also, from the parasite, the number of significantly regulated *N. ceranae* genes at multiple time points between the infection group and the siRNA-Dicer group were significantly deviated from random. We conclude that differentially expressed genes in either host or parasite were due to siRNA-Dicer feeding, which influenced parasite proliferation and host responses. Although RNAi functional assays can reveal nonspecific host responses, we feel our use of 25-nucleotide siRNAs and of a control treatment with a proportional but scrambled sequence helped ensure the targeting of specific transcripts. The decreasing group sizes during the infection can have effects on stress and disease, albeit mostly at the extremes (one bee vs. a small group vs. something closer to a colony unit). In our case, all treatment groups have the same sampling approach. This should not have biased the gene expression results.

### *Effects of siRNA-Dicer feeding on the host*

In order to interrupt or delay *N. ceranae* proliferation, siRNA-Dicer feeding might reverse the expression pattern of

honeybees' genes manipulated by *N. ceranae* during the infection. In this study, 150 honeybee genes were significantly regulated while spore proliferation was significantly delayed. From the physiological point of view, numerous enteric microorganisms colonize in honeybee guts and the honeybees need to protect their intestinal tract from the infection (Powell *et al*., 2014). Mucins, a family of high molecular weight, heavily glycosylated proteins with chitin binding domain, are the major component of the mucus barrier secreted by epithelial cells for intestinal protection (Gendler and Spicer, 1995; McGuckin *et al*., 2011). Just like antimicrobial peptides, mucin genes are regulated by host innate immunity (Kim and Khan, 2013). In order to reach the epithelium cells, the protozoan parasite *Giardia duodenalis* disturbs the integrity of mucus by proteolytic activity (Amat *et al*., 2016). Secretion of cysteine and serine protease is an additional strategy for parasites to overcome the mucus barrier (Lidell *et al*., 2006; Hasnain *et al*., 2012). For *N. ceranae*, the polar tube is the apparatus to inject the sporoplasm into the epithelium cells (Higes *et al*., 2007). So far, it is unclear whether *N. ceranae* infection destroys the honeybee mucus barrier. From our study, the honeybee gene *mucin-2-like* was significantly overexpressed in the siRNA-Dicer group compared with the infection group at 4, 5 and 6 dpi, which suggests siRNA-Dicer feeding promoted the strength (or renewal) of the mucus barrier resulting from interrupted parasite proliferation. *N. ceranae* has been repeatedly reported as suppressing the apoptosis of the infected honeybees, and a honeybee strain selected for tolerance of *N. ceranae* can escape the apoptosis manipulation (Higes *et al*., 2013; Kurze *et al*., 2015; Martín-Hernández *et al*., 2017). From our data, one apoptosis-associated gene, *tubulin alpha chain-like* (LOC552766), was found, which is cleaved by cytolytic serine protease granzume B to activate the apoptosis (Goping *et al*., 2006). The gene *tubulin alpha chain-like* was significantly upregulated in the infection group compared with the siRNA-Dicer group at 2 dpi. However, this gene was then significantly downregulated in the infection group compared with the siRNA-Dicer group at 5 dpi. Interestingly, a reversed expression pattern was found when comparing the infection group and uninfected group. Furthermore, an upregulation of apoptosis inhibitor was found in honeybees infected with *Nosema apis* and *N. ceranae*, which may play an essential role in reducing the apoptosis of the infected cells (Martín-Hernández *et al*., 2017). From our data, the apoptosis inhibitor KKO75779.1 of the *N. ceranae* genome was also found to be downregulated in the siRNA-Dicer group compared with the infection group. Additional histology analysis is required to conclude whether the siRNA-Dicer feeding promoted the apoptosis of the infection cells, as found from a tolerant honeybee population (Kurze *et al*., 2015).

*Effects of siRNA-Dicer feeding on the parasite*

SiRNA-Dicer feeding showed substantial impacts on the global gene expression profile of the parasite, with over 10% of the protein coding genes being significantly regulated. The function of those significantly regulated genes covered metabolism, DNA replication, DNA repair, RNA processing, protein processing and cell signalling. The transcription factor E2F3, which regulates cell proliferation from the G1 to S phases, was significantly downregulated in the siRNA-Dicer group at 3 dpi. The overexpression of E2F3 was associated with human cancer, and the E2F3 mutant cell showed defect cell proliferation (Humbert *et al*., 2000; Feber *et al*., 2003). A few more cell-proliferation-associated genes (DACAPO, E2F2 and Cyc B1) were overexpressed in honeybees infected with *N. ceranae* and *N. apis* compared with uninfected honeybees (Martín-Hernández *et al*., 2017). Those genes regulate the G1 to S phase progression. It seems that *Nosema* infection promotes the progression from the G1 phase to the S phase and that siRNA-Dicer feeding reduced the progression. The gene serine/threonine protein kinase, which regulates cell proliferation from the G2 phase to the M phase, was also significantly downregulated in the siRNA-Dicer group at 3 dpi. Total deletion of the gene serine/threonine protein kinase in bacteria significantly reduced development and spore formation (Hanlon *et al*., 1997). The spore wall is important to protect the spores from the environment for long-term survival (Bohne *et al*., 2011). The spore wall proteins also interacted with polar tube protein, which is essential for infection of the host (Li *et al*., 2012). For *N. ceranae*, five spore wall protein-coding genes were annotated from the genome. Two spore wall protein-coding genes were found continually significantly regulated in this study. Particularly at 3 dpi, both spore wall genes were significantly downregulated, which manifested interrupted spore proliferation. The enzyme hexokinase was reported as a virulent gene in the microsporidian parasite *Nematocida*, which accelerates the host metabolism to fuel fast parasite proliferation (Cuomo *et al*., 2012). In our study, the expression of hexokinase was not detected in the siRNA-Dicer group compared with the high expression levels in the infection group at 3 dpi, reflecting a decreased ability to manipulate host metabolism. The aforementioned evidence suggests that the proliferation of *N. ceranae* was reduced in the siRNA-Dicer group at least at 3 dpi.

*Gene co-expression between host and parasite*

Host–parasite co-evolution is a reciprocal genome adaptation of two antagonist species (Woolhouse *et al*., 2002; Rabajante *et al*., 2016). When the parasites infect the host, the host responds and defends. Then the parasites need to escape the host defence to proliferate

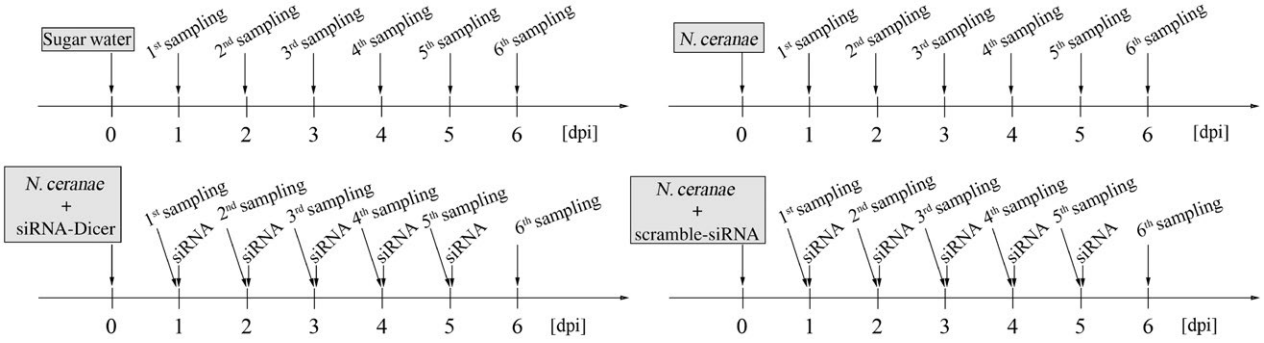


**Figure 5.** The experimental design of this study. Honeybees fed with sugar water were used to control for parasite contamination as the uninoculated group to determine spore contamination. The honeybees fed with *Nosema ceranae* spores were used to reflect the small interfering RNA (siRNA) treatment effect (ie the infection group). The honeybees fed with *N. ceranae* plus siRNA targeting the gene *Dicer* were used to quantify its effect on gene expression (both host and parasite itself) and overall parasite growth (ie the siRNA-Dicer group). The siRNA-Dicer group was fed at 24 h intervals for six times. The honeybees fed with *N. ceranae* plus scrambled siRNA were used to test the effect of foreign siRNA on gene expression (both host and parasite itself) and overall parasite growth (ie the siRNA-scramble group). The siRNA-scramble group was also fed at 24 h intervals for six times.

and transmit. Host–parasite interactions are studies from genotype$_{host}$–genotype$_{parasite}$ level and correlated gene expression level (Tierney *et al*., 2012; Reid and Berriman, 2013; Barribeau *et al*., 2014). In our study, two clusters of genes showed co-expression within both host and parasite. By reciprocal gene co-expression network analysis, two subsets of clusters showed co-expression within and between the host and parasite. While the prediction was that few host genes would be co-expressed with one cluster of parasite genes, the results were much more complicated. Indeed, we found host genes were co-expressed with parasite genes. However, the co-expression between host and parasite is not one cluster to one cluster, but one to multiple and multiple to one (Fig. 5). These results suggest that the host responds to the parasite via multiple routes to activate the same group of genes, and vice versa for the parasite. The host genes mainly fell into two clusters involved in various distinctive metabolism pathways. In contrast, the parasite genes' functions were quite diverse, not only with various metabolism pathways, but also with transcription, translation, repair, cell growth and death pathways. The gene hexokinase was significantly co-expressed for honeybees, partly explaining how honeybee metabolism pathways respond to the infection. Additionally, the parasite spore wall proteins, polar tube proteins and ABC transporters, which are associated with infection, were also co-expressed with honeybee genes (Paldi *et al*., 2010; Yang *et al*., 2015). Parasite genes, which were significantly regulated at multiple time points, showed a higher tendency to be co-expressed with honeybee genes than were genes regulated at a single time point ($P < 0.001$, Fisher's exact test). The results suggest that the co-expression of host metabolic genes and the *N. ceranae* genes that impact host metabolism are key constants during infection.

## Experimental procedures

### *Parasite infection and siRNA feeding*

*N. ceranae* spores were isolated from the midguts of heavily infected honeybees from multiple colonies. Spores were purified using a Percoll gradient procedure (Chen *et al*., 2013). Spores were counted using a Fuchs–Rosenthal haemocytometer, and *N. ceranae* species status was verified by species-specific PCR (Schwarz and Evans, 2013). Sealed brood frames were collected from multiple colonies and kept in an incubator (34 ± 1 °C, 60% relative humidity). The experiment consisted of four groups: (1) the uninfected group, consisting of 80 newly emerged workers that were individually fed with 2 µl sucrose solution without spores; (2) the infection group, consisting of an additional 80 newly emerged workers that were individually fed with 2 µl 50% sucrose solution containing $10^5$ *N. ceranae* spores without siRNA treatment; (3) the siRNA-Dicer group, consisting of 80 newly emerged workers that were individually fed with 2 µl 50% sucrose solution containing $10^5$ *N. ceranae* spores and 1.5 µg siRNA targeting parasite gene *Dicer*; and (4) the siRNA-scramble group, consisting of 80 newly emerged workers that were individually fed with 2 µl 50% sucrose solution containing $10^5$ *N. ceranae* spores and 1.5 µg scrambled siRNA (Fig. 5). Honeybees of siRNA-Dicer and siRNA-scramble groups were fed with their respective siRNAs to each honeybee individually for six times at 24 h intervals. In order to include cage effects, each group ($N = 80$) was split into two rearing cups (Evans *et al*., 2009). Each group of 40 honeybees was housed in a sterile plastic cup at 34 ± 1 °C, 60% relative humidity. Sugar water (50%) was provided *ad libitum* as the only food source. The sequences of the designed siRNAs are available from our previous study (Huang, *et al*., 2016a). The chance of being off-target/nonspecific is low, because the siRNAs did not require a further endonuclease process. The siRNA directly aligned to the gene *Dicer* for degradation. By blasting the sequence of the designed siRNAs, there is no significant hit in either host or parasite genome other than the expected gene *Dicer*. The detailed siRNA design and sequences have been described in a previous study (Huang, *et al*., 2016a).

### *Sample collection*

Five living honeybees from each cup were collected from 1 to 6 dpi at 24 h intervals to cover a complete parasite proliferation cycle. Total RNA was extracted from the midgut tissues of each of these five honeybees individually using TRIzol and then equal amounts of RNA were pooled for mRNA sequencing. Two sequencing libraries were prepared for each treatment at each sampling point. After 6 dpi, surviving honeybees were collected individually to count spores.

### *mRNA sequencing analysis and gene functional analysis*

On average, 58 million reads (114 nucleotide per read, pair end library) were generated from each mRNA library after trimming and quality control using FASTQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and SEQTK (https://github.com/lh3/seqtk). The mRNA sequencing reads were aligned to honeybee (Amel 4.5) and *N. ceranae* (ASM98816v1) genomes with the TOPHAT2 package (Kim *et al*., 2013). In order to assess the misassignment of the reads between the host and parasite, we realigned the reads that previously mapped to the host back to the parasite genome, and vice versa. The variance of two replicates was used to calculate significantly regulated genes with the EDGER package and adjusted for multiple comparisons (R Development Core Team, 2008; Robinson *et al*., 2010). The genes with normalized gene count less than 20 cpm in both replicates were removed. For the honeybees, the gene expression levels were pair-wise compared among *N. ceranae* infection group, siRNA-Dicer group and siRNA-scramble group. The significantly expressed honeybee genes due to siRNA-Dicer feeding must meet three criteria: (1) the genes are significantly differently expressed between the infection group and the siRNA-Dicer group; (2) the genes are significantly differently expressed between the siRNA-Dicer group and the siRNA-scramble group; and (3) the genes are not significantly differentially expressed between the siRNA-scramble group and the infection group. For *N. ceranae*, the gene expression levels were also pair-wise compared among the infection group, siRNA-Dicer group and siRNA-scramble group. The significantly differentially expressed *N. ceranae* genes due to siRNA-Dicer feeding must also meet the same three criteria as defined above. Furthermore, time series expression patterns of *N. ceranae* were compared in pairs to determine any changes of genes expression profile during the reproduction cycle using the MASIGPRO package (R Development Core Team, 2008). The co-expressed genes within and between honeybees and *N. ceranae* were clustered with the WGCNA package (Langfelder and Horvath, 2008). The protein sequences of candidate genes were used to query Pfam, Uniprot and KEGG databases by BLAST to retrieve the putative functions and pathways involved, and the Gene Ontology enrichment analysis was conducted with the dcGO database (Kanehisa and Goto, 2000; R Development Core Team, 2008; Fang and Gough, 2013; The Uniprot Consortium, 2017).

### *Statistics*

All statistical analyses were conducted with NCSS package. The overall mapped reads of different treatments across the reproduction cycle were compared among treatment groups with ANOVA. The treatments (infection group, siRNA-Dicer group, siRNA-scramble group) and postinfection days (1–6 dpi) were defined as fixed variable factors. The cups were defined as random variable factors. The distributions of differentially expressed honeybee genes were tested using the chi square test. Honeybee genes that were significantly expressed over more than one time point were compared with random using Fisher's exact test. The distributions of shared significantly differentially expressed genes over different time points were analysed with contingency tables. A correlation analysis of two replicates was performed for each treatment at each sampling point using Pearson correlation. The spore loads among the three treatment groups were analysed with Kruskal–Wallis one-way ANOVA. All the multiple comparisons were corrected for false discovery rate.

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## Conflict of interest

The authors declare no competing interests.

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## Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web site:

**Figure S1.** *N.* ceranae transcriptome expression data.

**Figure S2.** Honey bee transcriptome expression data.

**Figure S3.** The expression levels of *N.* ceranae and honey bee co-expressed genes.