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RESEARCH ARTICLE

# Nosemosis control in European honey bees, *Apis mellifera*, by silencing the gene encoding *Nosema ceranae* polar tube protein 3

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## ABSTRACT

RNA interference (RNAi) is a post-transcriptional gene silencing mechanism triggered by double-stranded RNA (dsRNA) that is homologous in sequence to the silenced gene and is conserved in a wide range of eukaryotic organisms. The RNAi mechanism has provided unique opportunities for combating honey bee diseases caused by various parasites and pathogens. *Nosema ceranae* is a microsporidian parasite of European honey bees, *Apis mellifera*, and has been associated with honey bee colony losses in some regions of the world. Here we explored the possibility of silencing the expression of a *N. ceranae* putative virulence factor encoding polar tube protein 3 (*ptp3*) which is involved in host cell invasion as a therapeutic strategy for controlling *Nosema* parasites in honey bees. Our studies showed that the oral ingestion of a dsRNA corresponding to the sequences of *N. ceranae ptp3* could effectively suppress the expression of the *ptp3* gene in *N. ceranae*-infected bees and reduce *Nosema* load. In addition to the knockdown of *ptp3* gene expression, ingestion of *ptp3*-dsRNA also led to improved innate immunity in bees infected with *N. ceranae* along with an improvement in physiological performance and lifespan compared with untreated control bees. These results strongly suggest that RNAi-based therapeutics hold real promise for the effective treatment of honey bee diseases in the future, and warrant further investigation.

**KEY WORDS: RNAi, Nosemosis, Polar tube protein, Silencing, Therapeutic treatment**

## INTRODUCTION

Pollinators play a vital role in the sustainability of ecosystems and biodiversity (Gallai et al., 2009; Potts et al., 2016). The European honey bee (*Apis mellifera*) is the most managed and widely used insect pollinator that provides important pollination services to a wide variety of food crops and plants in our agricultural and ecological system with enormous economic value (Gallai et al., 2009; Calderone, 2012). However, honey bee colony losses have been a growing concern and pose a significant challenge for the sustainability of our food production systems (Potts et al., 2016). While multiple factors including parasites, pathogens, pesticide residues, forage losses and poor nutrition have been proposed to explain colony losses, diseases caused by pathogens and parasites have been more often implicated in the decline of honey bee populations and health (Le Conte et al., 2010; Spivak et al., 2011; Cornman et al., 2012; Dainat et al., 2012; Vanbergen, 2013; van Engelsdorp et al., 2013; Goulson et al., 2015).

*Nosema ceranae* is an intracellular obligate microsporidian parasite that was first described in the Asian honey bee *Apis cerana*, and then later identified as a disease agent in the European honey bee, *A. mellifera* in 2006 (Fries, 2010; Holt and Grozinger, 2016). Since its emergence as a disease agent in *A. mellifera*, *N. ceranae* has dispersed around the world (Cox-Foster et al., 2007; Klee et al., 2007; Paxton et al., 2007; Chen et al., 2008; Invernizzi et al., 2009; Traver et al., 2012; Higes et al., 2013). Although there are considerable uncertainties regarding infection dynamics of *N. ceranae* in *A. mellifera* colonies, *N. ceranae* has been implicated in honey bee colony losses (Martín-Hernández et al., 2018).

*Nosema* infection begins when a bee ingests spores via fecal–oral and oral–oral transmission (Bailey, 1952; Smith, 2012). The spores germinate in the midgut lumen and inject their sporoplasm into epithelial cells with their polar tube (Fries et al., 1992; Kurze et al., 2015). The sporoplasm matures to a meront, which replicates several times giving rise to sporonts found in the primary spore stage. The same cycle can be repeated in the same cell or adjacent cells and finally gives rise to environmental spores (Fries et al., 1992; Gisder et al., 2011). Mature spores are liberated into the lumen via cell lysis where they may infect neighboring cells or may be excreted via defecation (Fries, 2010). Heavily infected worker honey bees can contain an excess of 50 million spores (Bailey, 1952; Forsgren and Fries, 2010).

Fumagillin, an antibiotic isolated from the fungus *Aspergillus fumigatus*, is the only registered chemical treatment for the control of *Nosema* disease. It has been extensively used in apiculture in the USA for more than 50 years for the treatment of *Nosema* infection in honey bees (Williams et al., 2008; Higes et al., 2011). This is not the case for Fumidil B in the UK, with fumagillin bicyclohexylamine salt as the active ingredient. The use of fumagillin in Europe is forbidden as it has no established maximum residue level (MRL; Commission Regulation, EU, 2010, no. 37/2010). With the prolonged use of fumagillin in USA, the issue of disease resistance to treatment has been a problem (Huang et al., 2013). As a result of the increasing prevalence of this pathogen (Zhu et al., 2014), additional therapeutic options are urgently needed for the treatment of *Nosema* disease in honey bees.

RNA interference (RNAi) is a natural mechanism for post-transcriptional gene silencing by double-stranded RNA (dsRNA) that is homologous in sequence to the silenced gene and has been

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used to manipulate gene expression in several organisms (Fire et al., 1998). RNAi-mediated gene knockdown has been widely used for controlling pest insects and investigating the functional role of specific genes in many insect species (Yu et al., 2012; Zhang et al., 2017). RNAi has also demonstrated a promising therapeutic modality for disease control in honey bees. Research has shown that ingestion of dsRNAs or siRNA that are complementary to honey bee RNA viruses including *Deformed wing virus* (DWV), *Israeli acute paralysis virus* (IAPV) and *Chinese sacbrood virus* (CSBV) leads to a significant reduction in virus titers within infected bees (Aronstein et al., 2006; Maori et al., 2009; Hunter et al., 2010; Chen and Evans, 2012; Desai et al., 2012; Chen et al., 2014; Zhang et al., 2016). The ectoparasitic mite, *Varroa destructor*, is the single most detrimental pest of the *A. mellifera*; it causes direct damage via feeding and vectoring of multiple viruses. Research has demonstrated that silencing several *Varroa* housekeeping transcripts leads to a significant reduction in the number of *Varroa* mites within honey bee colonies (Campbell et al., 2010; Garbian et al., 2012). Previous studies with *N. ceranae* showed that silencing both *Nosema* virulence factors and a host immune suppressor reduces parasite load, activates honey bee immune responses, and improves the overall health of infected honey bees (Paldi et al., 2010; Li et al., 2016). Therefore, the search for new target genes in *N. ceranae* will improve the knowledge of this pathogen and the design of new treatments.

Inspired by our successes in earlier work using RNAi to mitigate *Nosema* disease infection in honey bees, we further explored the potential of RNAi-based gene silencing of key genes that are required by *Nosema* for host invasion and disease control. Like most microsporidia parasites with an obligate intracellular lifestyle, *N. ceranae* spores contain a unique extrusion apparatus, the polar tube, which delivers sporoplasm into host cells during invasion. Molecular characterization of microsporidian parasites has resulted in the identification of three polar tube proteins: a proline-rich ptp1, a lysine-rich ptp2, and a ptp3 with a molecular mass of >135 kDa, which form the polar tube structure (Xu and Weiss, 2005). The genome sequence analysis of *N. ceranae* and *N. apis* confirmed the presence of homologous polar tube proteins in *Nosema* (Chen et al., 2009; Cornman et al., 2009). The genome analysis of *N. ceranae* reveals that, of the three conserved ptps identified in another microsporidia species, only ptp3 was identified in *N. ceranae* (Cornman et al., 2009). In this work, we selected the *N. ceranae* polar tube protein 3 (ptp3) which is one of the three essential components of the polar filament in *N. ceranae* and other microsporidia (Peuvel et al., 2002) as a target gene for knockdown. We demonstrated that the oral ingestion of dsRNA corresponding to the region of *ptp3* could lead to specific gene silencing, *Nosema* load reduction, improvement of host physiology, and extension of lifespan in infected bees. These results suggest that RNAi-based treatment could be an effective and specific tool for the control of diseases in bees.

## MATERIALS AND METHODS

### Ethics statement

The experimental honey bee colonies that were originally from commercial packages were reared in the Bee Research Laboratory apiaries. The apiaries are the property of the United States Department of Agriculture–Agricultural Research Service (USDA-ARS) and are not privately owned or protected in any way. Studies involved the European honey bee (*Apis mellifera*), which is neither an endangered nor a protected species.

### Honey bees

Honey bees were collected from colonies of *Apis mellifera ligustica* Spinola 1806 maintained at the USDA-ARS Bee Research Laboratory apiaries, Beltsville, MD, USA. The study was conducted in June and July, 2017. The frames with sealed brood from healthy colonies that were identified as *Nosema*-negative by our monthly disease survey (Cantwell, 1970) were removed from bee colonies without nurse bees, and placed in a mesh-walled cage individually and stored in an insect growth chamber at 34±1°C and 55 ±5% relative humidity (RH) overnight. After 24 h to allow newly emerged bees to roam on whole frame consisting of brood, pollen and honey to acquire gut microbiota, we collected the newly emerged worker bees for the subsequent *N. ceranae* inoculation. The negative status of *N. ceranae* infection of newly emerged bees was further confirmed using a hemocytometer and light microscopy following a previously described method (Cantwell, 1970), to make sure that the bees used in our experiment were free of *N. ceranae* infection before conducting the experimental inoculation. The bees were starved for at least 2 h before the subsequent *Nosema* inoculation.

### Preparation of spore inoculum

Forager honey bees were collected with an insect vacuum outside the hive entrance from colonies that were identified as *N. ceranae*-positive. Around 300 midguts were pulled out from the bees and homogenized in sterile, distilled water. The homogenate was filtered through a nylon mesh cloth (65 µm pore size) by centrifugation for 5 min at 3000 ***g***. The supernatant was discarded and the spore pellet was suspended in sterile water and centrifuged for 10 min at 5000 ***g***. After the supernatant was discarded, the spores were washed twice more. The spores were diluted to a final concentration of $2.0\times10^7$ spores $ml^{-1}$ in 50% (w/v) sucrose solution confirmed using the Neubauer chamber spore count as suggested by the World Organisation for Animal Health (http://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.02.04_NOSEMOSIS_FINAL.pdf). Spores were stored at 4°C until use.

### Individual infection

Individual starved bees, taken from the same randomized pool, were fed with 5 µl inoculum (100,000 spores per bee) by holding onto the wings of a bee with one hand and feeding the bee with solution using a 10 µl pipettor and then transferring the bee to a top-feeder rearing cage (Evans et al., 2009; Huang et al., 2014). Thirty-five inoculated bees were transferred randomly to each rearing cage. Uninfected control bees were fed with only 5 µl 50% sucrose syrup.

### Primer design

Knockdown primers were designed from the sequence of the *N. ceranae ptp3* that share highly conserved sequences across different isolates in GenBank (NcORF-00083, GenBank accession no. XM_002996713.1; Table 1), and without matches to non-target genes. The primers sequences included the T7 promoter sequence (5′-taa tac gac tca cta tag ggc ga-3′) using the E-RNAi web service (Horn and Boutros, 2010). Quantitative polymerase chain reaction (qPCR) primers were designed using the primer 3 web tool (http://bioinfo.ut.ee/primer3-0.4.0/).

### dsRNA synthesis

To obtain the *N. ceranae* DNA, an aliquot of spore solution was disrupted for 45 s at 65 m $s^{-1}$ speed using a FastPrep cell disrupter in a 2.0 ml tube containing sterile 1.4 mm zirconium silicate grinding beads (Quackenbush Co., Inc., Crystal Lake, IL, USA) and 400 µl CTAB buffer (100 mmol $l^{-1}$ Tris-HCl, pH 8.0, 20 mmol $l^{-1}$ EDTA,

Journal of Experimental Biology (2018) 221, jeb184606. doi:10.1242/jeb.184606

**Table 1. Primer sequences used in this study**

| Purpose | Gene name | Primer | Sequence (5′→3′)* | Reference |
|---|---|---|---|---|
| dsRNA | *ptp3* | Nc83-F | taatacgactcactatagggcgaAGAATGGATTTGGCTGATGC | This study‡ |
| | (*N. ceranae*) | Nc83-R | taatacgactcactatagggcgaTTCAGCCACCAAATGTGCTA | |
| qPCR | *ptp3* | qPCR-Nc83-F | AGCACAAGGAGTCGAGCAAA | This study§ |
| | (*N. ceranae*) | qPCR-Nc83-R | TGCTGCCTCAAATCCTACCT | |
| | *16S rRNA* | rRNA-F | CGGATAAAAGAGTCCGTTACC | Chen et al. (2008) |
| | (*N. ceranae*) | rRNA-R | TGAGCAGGGTTCTAGGGAT | |
| | *β-actin* | β-actin-F | ATGCCAACACTGTCCTTTCTGG | Yang and Cox-Foster (2005) |
| | (*A. mellifera*) | β-actin-R | GACCCACCAATCCATACGGA | |
| | *Abaecin* | Abaecin-F | CAGCATTCGCATACGTACCA | Evans et al. (2006) |
| | (*A. mellifera*) | Abaecin-R | GACCAGGAAACGTTGGAAAC | |
| | *Hymenoptaecin* | Hym-F | CTCTTCTGTGCCGTTGCATA | Evans (2006) |
| | (*A. mellifera*) | Hym-R | GCGTCTCCTGTCATTCCATT | |
| | *Defensin-1* | Defensin-F | TGTCGGCCTTCTCTTCATGG | Li et al. (2016) |
| | (*A. mellifera*) | Defensin-R | TGACCTCCAGCTTTACCCAAA | |
| | *Apidaecin* | Apidaecin-F | TTTTGCCTTAGCAATTCTTGTTG | Li et al. (2016) |
| | (*A. mellifera*) | Apidaecin-R | GCAGGTCGAGTAGGCGGATCT | |
| | *birc5* | birc5-F | CTTCTGACAATTCGTGCAATCC | Martín-Hernández et al. (2017) |
| | (*A. mellifera*) | birc5-R | GGGTTCTTTCTTACCACCCACTAC | |

Gene name is followed by the species in parentheses. *Underlining shows the T7 promoter sequence. ‡Sequence length, 489 base pairs; §sequence length, 100 base pairs.

pH 8.0, 1.4 mol $l^{-1}$ sodium chloride, 2% cetyltrimethylammonium bromide, 0.2% 2-mercaptoethanol) with proteinase K (200 µg $ml^{-1}$), then incubated overnight at 50°C. One milliliter of DNAzol reagent was added and mixed using the FastPrep disrupter again. The homogenate was centrifuged for 10 min at 10,000 ***g***. The supernatant was transferred to a new tube with 500 µl of ethanol absolute and incubated for at least 30 min at −20°C. The suspension was centrifuged for 30 min at 10,000 ***g*** and the pellet was washed with 70% ethanol. The resultant pellet was resuspended in sterile water.

Conventional PCR reactions were performed for each gene individually to obtain DNA template. The 100 µl PCR reaction mixture contained the following components: 78 µl $H_2O$, 10 µl 10× reaction buffer (Invitrogen), 3 µl $MgCl_2$, 2 µl dNTP mix (10 mmol $l^{-1}$, Invitrogen), 2 µl forward primer (20 µmol $l^{-1}$), 2 µl reverse primer (20 µmol $l^{-1}$), 1 µl Taq polymerase (Invitrogen) and 2 µl DNA template. The thermal profile of the PCR amplification was as follows: one cycle of 94°C for 3 min followed by 35 cycles of 94°C for 30 s, 56°C for 30 s and 72°C for 90 s with a final extension of 72°C for 10 min. After PCR amplification, gel electrophoresis in 1.0% agarose gels were performed to verify the expected target. The purified PCR products were then used as templates for the *in vitro* transcription reaction. The dsRNA was synthesized using the MEGAscript RNAi Kit (Ambion) following the manufacturer's instructions. Briefly, the transcription reactions were assembled and the incubation time was extended to 15 h at 37°C. The products of dsRNA were verified in 1.0% agarose gels and the concentration of dsRNA was determined with a spectrophotometer (NanoDrop 8000, Thermo Fisher Scientific). A previous dose-dependent assay was performed to determine the concentration of *ptp3*-dsRNA (20, 40, 60 and 80 ng $ml^{-1}$) demonstrating the greatest efficacy with respect to spore count reduction (data not shown). The dsRNA was diluted in 50% sucrose solution to obtain a 40 ng $ml^{-1}$ concentration, and was stored at −80°C until used.

## dsRNA treatment

Three groups were created in this study: group I, *Nosema*-infected bees+*ptp3*-dsRNA treatment; group II, *Nosema*-infected bees; and group III, healthy bees (non-infected, non-treatment). The treated group was fed with 40 ng $ml^{-1}$ dsRNA (*ptp3*-dsRNA) *ad libitum* from a 3 ml syringe. The same feeding procedure was carried out with the untreated bees but fed only with sugar solution. All groups were supplied with a piece of pollen patty in the bottom of the cage to provide complete protein nutrition. All cup cages were maintained in an insect incubator (32°C and 75% RH). All groups were run at same time.

## Data collection

Each group consisted of seven cages (35 bees per cage); four of them were used for spore counts and molecular analysis, and the other three to evaluate daily mortality and consumption. The amount of syrup consumed was measured daily following the graduation of the top feeding syringe, and the sucrose solution was renewed every 2 days. The pollen patty was supplied and changed every 3 days. The number of dead bees was recorded and removed

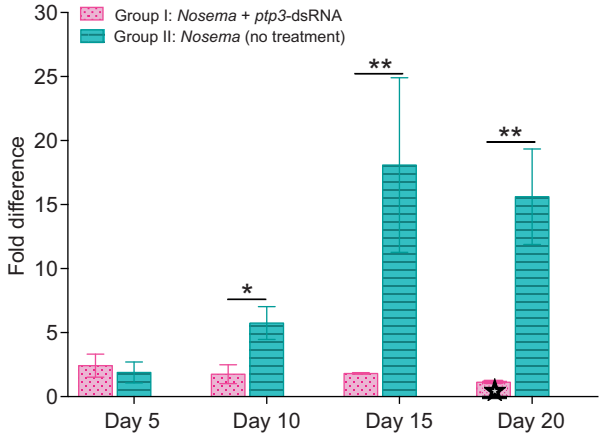


**Fig. 1. Dynamic of *Nosema ceranae ptp3* expression in infected bees at different sampling points.** Honey bees were inoculated with $10^6$ *N. ceranae* spores first and then fed with *ptp3*-dsRNA (group I), or negative control without any treatment (group II). The fold difference is expressed as mean±s.d. The calibrator for each time interval used to normalize the gene expression was the group with the lowest expression and is represented with a grey star. A *t*-test was used to analyse the differences between data, and represented with asterisks (*$P$<0.05, **$P$<0.001). Group III had no *N. ceranae* spores and was not represented.

Journal of Experimental Biology (2018) 221, jeb184606. doi:10.1242/jeb.184606

daily. At days 5, 10, 15 and 20 post-treatment, 24 bees were sampled individually (12 for spore counting and 12 for molecular analysis) and stored at −80°C until used.

### Spore counting

Twelve individual bees were collected from each group at each time interval to evaluate the *N. ceranae* infection level. The bees were individually placed into 1.5 ml tubes and homogenized thoroughly in 1 ml $dH_2O$ using a disposable pestle. Ten microliters of each bee solution (diluted 1:100) was loaded onto a hemocytometer and the number of spores was counted under a light microscope as described above. The spore load was obtained using the formula [(spore counts×100)/20]=spores per bee or per milliliter.

### RNA extraction and cDNA synthesis

The other 12 bees were collected and analysed separately from each group at each time interval. The bees were individually transferred into 2 ml tubes. Each tube contained sterile 1.4 mm zirconium silicate grinding beads (Quackenbush). One milliliter of TRIzol reagent (Ambion) was added to each tube to extract total RNA using FastPrep to disrupt the samples and then following the manufacturer's protocol. A treatment with DNase I (Invitrogen) was applied to remove any genomic DNA contamination. After extraction, all samples were examined using a NanoDrop 8000 spectrophotometer (Thermo Fisher Scientific) to determine the purity and quantity of RNA samples. All RNAs were stored at –80°C until use. First-strand cDNA was produced using a 20 µl

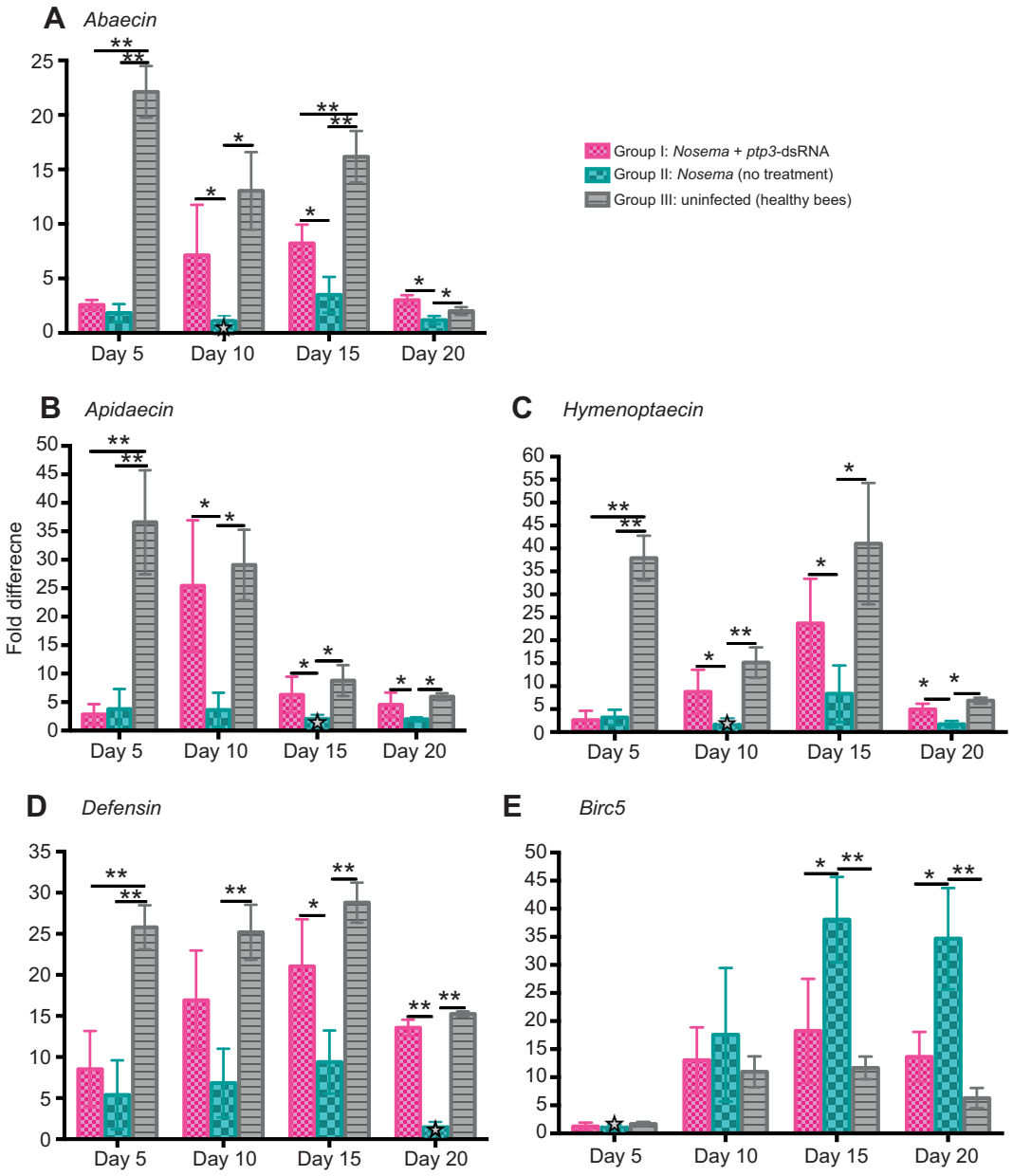


**Fig. 2. Effects of *ptp3*-dsRNA treatment on the expression of genes encoding *abaecin, apidaecin, hymenoptaecin, defensin* and *birc5* at days 5, 10, 15 and 20 post-treatment.** (A) *Abaecin*; (B) *apidaecin*; (C) *hymenoptaecin*; (D) *defensin-1*; (E) *birc5*. Group I, *Nosema*-infected bees with *ptp3*-dsRNA treatment; group II, *Nosema*-infected bees without treatment; group III, *Nosema*-uninfected healthy bees. The relative normalized expression is expressed as mean±s.d. The calibrator for each time interval used to normalize the gene expression was the group with the lowest expression and is represented with a gray star. One-way ANOVA and Tukey's post hoc test was used to analyse the differences between data. Significant differences are represented with asterisks (*$P$<0.05, **$P$<0.001).

reverse transcription reaction mixture following the manufacturer's recommendations with the SuperScript III Retrotranscriptase kit (Invitrogen). The cDNAs were stored at –20°C until use in qPCR.

### Quantitative real time PCRs

qPCRs were run on a CFX384 Touch Real-Time PCR System (Bio-Rad, Hercules, CA, USA), SYBR Green was selected as the detection signal, and *Apis mellifera* β-actin was used as a reference gene. The primers were designed with Primer3 (Table 1) (Untergasser et al., 2012). Each 10 µl of PCR mixture was assembled by mixing 5 µl 2× Brilliant III Ultra-Fast SYBR Green qPCR mix (Agilent Technologies), 0.25 µl forward primer (20 mmol $l^{-1}$), 0.25 µl reverse primer (20 mmol $l^{-1}$), 0.5 µl cDNA, and 4 µl nuclease-free water. Each reaction was run in triplicate, beside positive, negative and non-target (NTC) controls. The PCR program was 95°C for 3 min, followed by 40 cycles of 95°C for 10 s and 60°C for 45 s. Amplification efficiency and melting curves were monitored to evaluate the quality and specificity of amplification. The threshold cycle ($C_T$) values were generated using CFX Manager 3.1 (Bio-Rad). The relative quantification of gene expression was calculated with the comparative $C_T$ ($\Delta\Delta C_T$) method (Schmittgen and Livak, 2008). For each gene, the average $C_T$ value of the target was normalized with the corresponding β-actin value using the formula $\Delta C_T$=average $C_{T(target)}$ − average $C_{T(\beta\text{-actin})}$, and for each gene the group of bees with the lowest level of gene expression was chosen as the calibrator [$\Delta C_{T(calibrator)}$]. The $\Delta C_T$ value of each group was subtracted from the $\Delta C_{T(calibrator)}$ value to generate the $\Delta\Delta C_T$. The concentration of each target in each group was calculated using the formula $2^{-(\Delta\Delta C_T)}$ and expressed as the fold difference.

### Statistical analyses

Transcript levels of *N. ceranae ptp3* and *16S ribosomal RNA gene* (*rRNA*) and spore counts were analysed by *t*-test. The relative expression for each immune gene after dsRNA treatment and mortality were analysed by one-way analysis of variance (ANOVA, normality and variance homogeneity was checked in each analysis), and Tukey's post hoc test was used to determine the differences between groups. In all cases, a $P$ value of <0.05 was taken to be significant. All analyses were carried out using SPSS software 18.0 and GraphPad Prism (GraphPad Software, La Jolla, CA, USA).

## RESULTS

### Ingestion of *ptp3*-dsRNA silences the transcript level of *N. ceranae ptp3* gene in infected bees

The ingestion of *ptp3*-dsRNA knocked down *ptp3* gene expression in *N. ceranae*-infected bees (Fig. 1). Ten days after treatment, mRNA transcript level of the *ptp3* gene in group I fed with *ptp3*-dsRNA was significantly reduced, compared with group II of *N. ceranae*-infected bees without treatment. The silenced *ptp3* gene expression lasted for at least 20 days, and the difference in *ptp3* mRNA abundance was more significant at days 15 and 20, compared with day 10 (*t*-test: 5 days, $P$>0.05; 10 days, $P$<0.05; 15 days, $P$<0.001; 20 days, $P$<0.001). Uninfected bees (group III) were *Nosema*-negative for the whole test.

### Silencing of the *N. ceranae ptp3* gene boosts the immune responses of *Nosema*-infected bees

Several genes encoding immune peptides were used to evaluate the effect of *ptp3*-dsRNA treatment on *N. ceranae*-infected bees. The genes evaluated in the study consisted of antimicrobial peptide (AMP) genes (*Abaecin*, *Apidaecin*, *Hymenoptaecin* and *Defensin-1*), and a gene encoding an apoptosis inhibitor protein (IAP), baculoviral IAP repeat-containing 5 (*birc5*).

As shown in Fig. 2, the expression of the genes encoding immune peptides involved in the Toll pathway was significantly down-regulated in *N. ceranae*-infected bees compared with healthy uninfected bees. Treatment of *N. ceranae*-infected bees with *ptp3*-dsRNA could help reduce the immune suppression, contributing to the level of infection observed after day 10 post-treatment (Fig. 2A–D). The treatment of *N. ceranae*-infected bees with *ptp3*-dsRNA could also suppress the expression of *birc5*, whose expression was positively correlated with the level of *Nosema* titers in infected bees. Although there was no significant difference in antimicrobial peptide gene expression between the groups of *N. ceranae*-infected bees with *ptp3* treatment (group I) and without treatment (group II) at day 5, the expression of genes encoding *Abaecin*, *Defensin-1*, *Hymenoptaecin* and *Apidaecin*, in group I at day 10 post-treatment was significantly higher than in

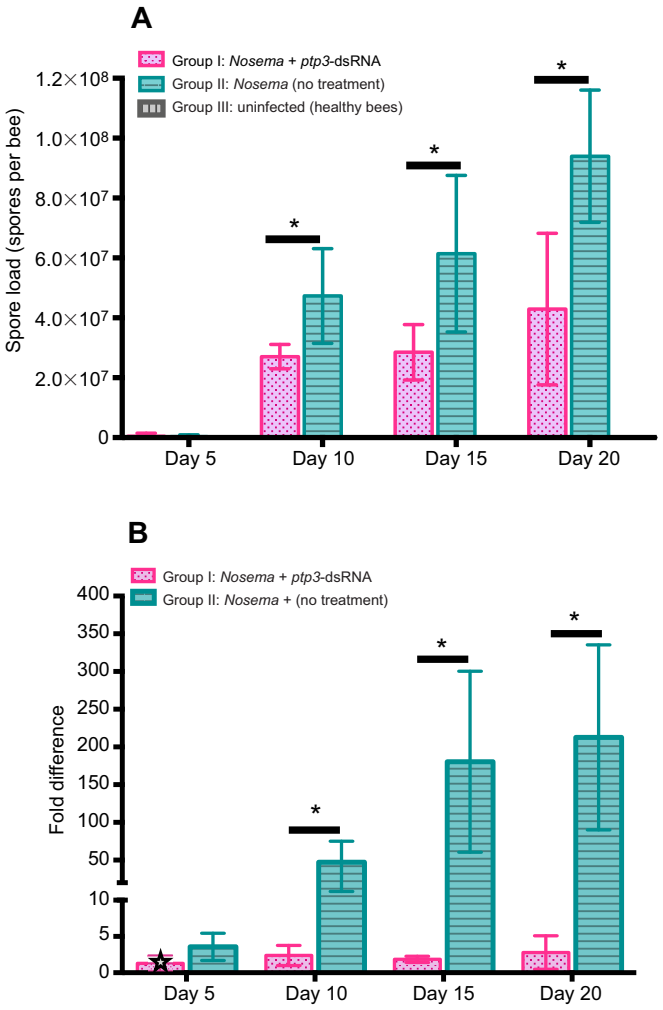


**Fig. 3. Effect of *ptp3* silencing on the spore load of adult workers infected by *N. ceranae*.** (A) The spore load is expressed as mean±s.d. (B) The relative normalized expression of 16S rRNA is also expressed as mean±s.d. The calibrator for each time interval used to normalize the gene expression was the group with the lowest expression and is represented with a gray star. A *t*-test was employed to analyse the differences between data, and represented with asterisks (*$P$<0.05, **$P$<0.001). Group III was free of spores and without 16S rRNA expression during all sampling points, so is not represented in B.

Journal of Experimental Biology (2018) 221, jeb184606. doi:10.1242/jeb.184606

bees from group II (ANOVA; Fig. 2A: *abaecin*, $F$=9.623, $P$<0.001; Fig. 2B: *apidaecin*, $F$=11.448, $P$<0.001; Fig. 2C: *hymenoptaecin*, $F$=10.730, $P$<0.001; Fig. 2D: *defensin-1*, $F$=18.929, $P$<0.001). In addition, after the *ptp3*-dsRNA treatment, the expression level of *birc5* in *N. ceranae*-infected bees treated with *ptp3*-dsRNA was significantly suppressed compared with *N. ceranae*-infected bees without treatment at days 15 and 20 (Fig. 2E: ANOVA, $F$=16.524, $P$<0.001).

### Silencing of *N. ceranae ptp3* gene leads to significant reduction of *N. ceranae* spore load in infected bees

At around 10 days of feeding, 40 ng ml$^{-1}$ of dsRNA was required to reduce *N. ceranae* development (Fig. 3A). The results obtained in *N. ceranae* spore counts at day 10 were correlated with the silencing of *N. ceranae ptp3* gene in group I (Fig. 1). Bees treated with *ptp3*-dsRNA had significantly fewer spores than the untreated group (Fig. 3A, *t*-test: 10, 15 and 20 days, $P$<0.05). In addition, the *N. ceranae* 16S rRNA gene levels supported the effectiveness of the silencing (Fig. 3B, *t*-test: 10, 15 and 20 days, $P$<0.05). As expected, group III was free of spores during all sampling points, indicating that there was no contamination during these experiments.

### Knockdown of the *N. ceranae ptp3* extends the lifespan of *N. ceranae*-infected bees

Mortality analysis was performed to examine the effect of silencing the *ptp3* gene on the lifespan of *Nosema*-infected honey bees. As shown in Fig. 4, the cumulative mortality among the three groups was significantly different (ANOVA: 5 days, $F$=1.195, $P$>0.05; 10 days, $F$=126.270, $P$<0.001; 15 days, $F$=38.41, $P$<0.001; 20 days, $F$=83.016, $P$<0.001). *Nosema*-infected bees in group II without treatment had the highest mortality, while healthy bees from group III, without *Nosema* infection, had the lowest mortality throughout the entire study period among the three experimental groups. At 15 days post-treatment, the bees from group I treated with *ptp3*-dsRNA exhibited lower mortality than bees in group II without treatment ($P$<0.05).

## DISCUSSION

The therapeutic potential of double-stranded RNA-mediated interference (RNAi) has been applied to reduce pest insect populations and control pathogenic diseases in insects (reviewed in Gundersen-Rindal et al., 2017). The complete genome analysis of *A. mellifera* divulged the existence of the genetic machinery involved in RNA silencing within this species. The availability of the genomes for *N. ceranae* and *N. apis* along with comparative genomic analysis of the two *Nosema* species (Cornman et al., 2009; Chen et al., 2013) have led to identification of virulence factors that are involved in adhesion, invasion, immune evasion, colonization, and replication of the intracellular parasite within honey bee hosts. Improved understanding of the life cycle and pathogenicity of *N. ceranae* during the infection process has enabled us to develop RNAi-based therapeutics for treatment of honey bee nosemosis (Paldi et al., 2010; Li et al., 2016).

All microsporidia possess a specialized invasion structure called the polar tube, which pierces a host cell membrane and serves as a bridge to deliver the infectious sporoplasm to the host cell. The polar tube is composed of three distinct polar tube proteins (ptps). A previous study hypothesized that *ptp3* is involved in the sporoblast-to-spore polar tube biogenesis and plays a role in the control of

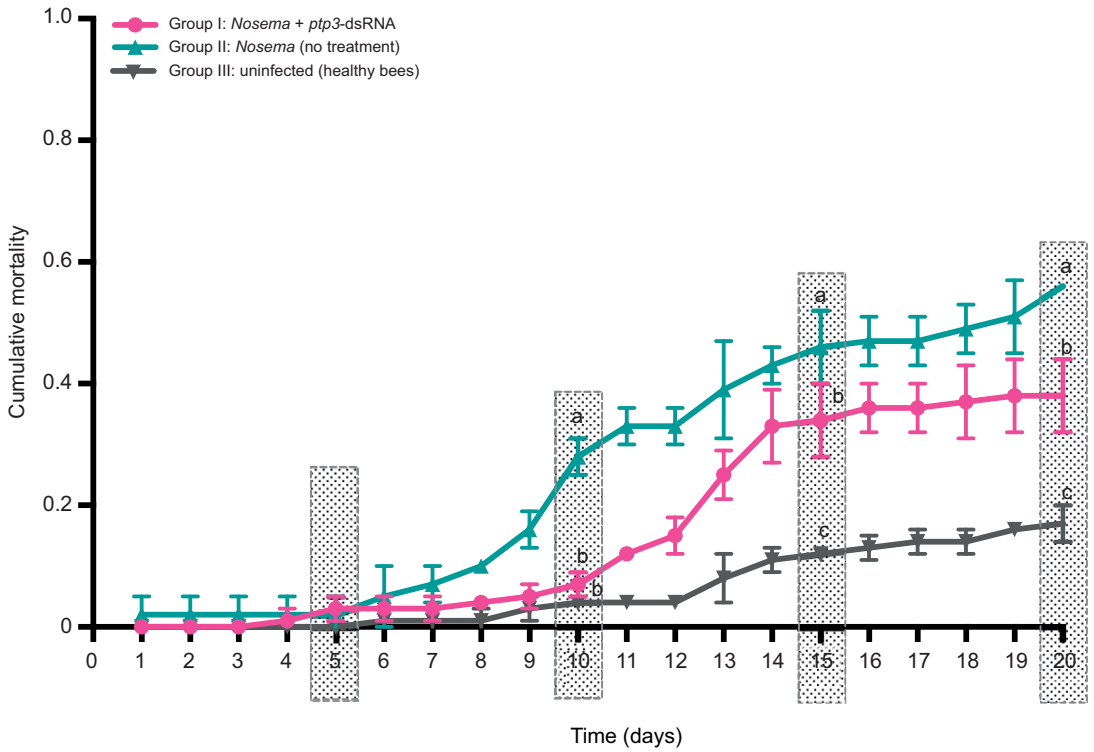


**Fig. 4. Cumulative mortality for the three experimental groups.** $N$=105 bees per group. Data represent the proportion of cumulative mortality (mean±s.d.) of the three groups over time (20 days). Group I, *Nosema*-infected bees treated with *ptp3*-dsRNA (pink line); group II, *Nosema*-infected bees without treatment (blue line); group III, healthy bees without *Nosema* infection (gray line). Shaded boxes show the days of mortality analysis. Knockdown of the *N. ceranae ptp3* gene reduced the incidence of death. Different letters denote significant differences ($P$<0.05 by ANOVA and Tukey's post hoc test).

the polar tube extrusion (Peuvel et al., 2002); we explored the therapeutic potential of silencing *ptp3* gene expression for controlling *N. ceranae* infection in honey bees.

Our results showed that ingestion of dsRNA corresponding to a region of *ptp3* gene could lead to the knockdown of *ptp3* gene expression in *N. ceranae*-infected bees starting at day 10 post-treatment. An earlier study showed that ingestion of dsRNA homologous to *N. ceranae* virulence factors ADP/ATP transporters, specifically silenced the transcripts encoding these proteins and inhibited *Nosema* replication in honey bees (Paldi et al., 2010). Given the fact that these two studies conducted across different bee populations yielded very similar results, we can be confident that silencing the virulence factors of the parasite by RNAi is a promising and specific way for controlling *Nosema* infection in honey bees.

In addition to the knockdown of *ptp3* gene expression, ingestion of *ptp3*-dsRNA also led to improved innate immunity in *N. ceranae*-infected bees. Previous studies reported that *N. ceranae* infection could lead to immune suppression in the infected honey bee (Antúnez et al., 2009; Chaimanee et al., 2012), suggesting a possible mechanism that is responsible for the establishment of widespread infection of *N. ceranae* in honey bee host populations. Our study confirms previous findings and shows that *N. ceranae*-infected bees (groups I and II) had a significantly lower level of expression of genes encoding immune peptides *Abaecin*, *Apidaecin, Hymenoptaecin* and *Defensin-1*, compared with the group III of healthy and uninfected bees. Our results show that the gene expression of *Apidaecin*, *Abaecin*, *Hymenoptaecin* and *Defensin-1* in *N. ceranae*-infected bees with *ptp3*-dsRNA treatment started to increase at day 10 post-treatment, suggesting that an alteration of the immune response, as a consequence of the treatment, could be contributing to the level of infection observed. While the level of immune gene expression declined after day 15 post-treatment in both *N. ceranae*-infected bees with *ptp3*-dsRNA treatment and healthy bees, probably due to stressors associated with restraining cages, the level of immune gene expression in group I (*Nosema*-infected bees with *ptp3* treatment) was still significantly higher than in group II (*Nosema*-infected bees without *ptp3* treatment).

Apoptosis, a physiological process of controlled cell death, is an integral part of all aspects of immune function and occurs in response to a variety of physiological and pathophysiological stimuli. Previous studies with the TUNEL (terminal deoxynucleotide transferase mediated X-dUTP nick end labelling) technique within gene expression analyses showed that *N. ceranae* inhibited the apoptosis of the cells which it parasitizes and has a negative impact on host immunity (Kurze et al., 2015; Huang et al., 2016; Martín-Hernández et al., 2017). The gene *birc5* (baculoviral IAP repeat-containing 5) is a member of the inhibitor of apoptosis gene family, which encode negative regulatory proteins that inhibit *caspase* activation, thereby inhibiting apoptotic cell death. In our study, although *N. ceranae*-infected bees without treatment (group II) showed an increase in the expression of *birc5*, in *Nosema*-infected bees with *ptp3*-dsRNA treatment (group I) the expression of *birc5* was significantly reduced, indicating that RNAi could be enhancing the innate immune response, allowing infected cells to undergo apoptosis (Martín-Hernández et al., 2017), further demonstrating the role of silencing the *ptp3* gene in boosting honey bee immunity.

Spore counts and qPCR analysis were not synchronized in time due to the sensitivity differences between both techniques, but both showed how the knockdown of the *ptp3* gene by feeding *Nosema*-infected bees with *ptp3*-dsRNA reduced the extent of *Nosema* infection significantly after *ptp3*-dsRNA treatment. The overall improvement of health after silencing the *ptp3* gene is shown by infected bees exhibiting an extended lifespan after treatment. In summary, all of the results from our study confirm that RNAi-based therapeutics are an effective approach to control *N. ceranae* infection in honey bees, and the *N. ceranae* gene *ptp3* is a good candidate for the development of a therapeutic strategy. The successes in previous works with RNAi technology under natural beekeeping conditions (Hunter et al., 2010) encourage the development of this technique against *N. ceranae* for large-scale field application in the future. Considering the widely demonstrated advantages of RNAi specifically targeting a gene product, we foresee that RNAi will become a powerful therapeutic approach for honey bee health improvement.

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#### Competing interests
The authors declare no competing or financial interests.

#### Author contributions
Conceptualization: C.R.-G., J.D.E.; Methodology: W.L., B.B., J.H.L., M.C.H., O.B., Y.Z., M. Hamilton, R.M.-H., Y.P.C.; Validation: C.R.-G., M. Hamilton; Formal analysis: C.R.-G., B.B., M. Hamilton, R.M.-H., Y.P.C.; Investigation: C.R.-G., W.L., B.B., J.H.L., M.C.H., O.B., M. Hamilton, R.M.-H., Y.P.C.; Resources: R.M.-H., Y.P.C.; Writing - original draft: C.R.-G.; Writing - review & editing: C.R.-G., J.D.E., W.L., B.B., J.H.L., M.C.H., M. Higes, Y.P.C.; Supervision: J.D.E., M. Hamilton, R.M.-H., Y.P.C.; Project administration: J.D.E., Y.P.C.; Funding acquisition: J.D.E., M. Higes, R.M.-H., Y.P.C.

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