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5 **Silencing honey bee (*Apis mellifera*) *naked cuticle (nkd)* improves host immune**  
6 **function and reduces *Nosema ceranae* infections**

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38 **ABSTRACT**

39 *Nosema ceranae* is a new and emerging microsporidian parasite of European honey  
40 bees, *Apis mellifera* that has been implicated in colony losses worldwide. RNA  
41 interference (RNAi), a post-transcriptional gene silencing mechanism, has emerged as  
42 a potent and specific strategy for controlling infections of parasites and pathogens in  
43 honey bees. While previous studies have focused on the silencing of parasite/pathogen  
44 virulence factors, here we explore the possibility of silencing a host factor as a  
45 mechanism for reducing parasite load. Specifically, we use an RNAi strategy to  
46 reduce the expression of a honey bee gene, *naked cuticle (nkd)* which is a negative  
47 regulator of host immune function. Our studies found that *nkd* mRNA levels in adult  
48 bees were upregulated by *N. ceranae* infection (and thus the parasite may use this  
49 mechanism to suppress host immune function), and ingestion of dsRNA specific to  
50 *nkd* efficiently silenced its expression. Furthermore, we found that RNAi-mediated  
51 knockdown of *nkd* transcripts in *Nosema*-infected bees resulted in upregulation of  
52 expression of several immune genes (*Abaecin*, *Apidaecin*, *Defensin-1*, and *PGRP-S2*),  
53 reduction of *Nosema* spore loads, and extension of honey bee lifespan. The results of  
54 our studies clearly indicate that silencing the host *nkd* gene can activate honey bee  
55 immune responses, suppress the reproduction of *N. ceranae* and improve the overall  
56 health of honey bees. This study represents a novel host-derived therapeutic for honey  
57 bee disease treatment that merits further exploration.

58 **IMPORTANCE**

59 Given the critical role of honey bees in the pollination of agricultural crops, it is  
60 urgent to develop strategies to prevent the colony decline induced by the infection of  
61 parasites/pathogens. Targeting parasites and pathogens directly by RNAi has been  
62 proved to be useful for controlling infections in honey bees but little is known about  
63 the disease impacts of RNAi silencing of host factors. Here, we demonstrate that  
64 knocking down the gene encoding the honey bee immune repressor *nkd* can suppress  
65 the reproduction of *N. ceranae* and improve the overall health of honey bees, which  
66 highlights the potential role of host-derived and RNAi-based therapeutics in  
67 controlling the infections in honey bees. The information obtained from this study  
68 will have positive implications for honey bee disease management practices.

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71 Honey bees, *Apis mellifera*, play a critical role in pollination of important crops.  
72 However, the honey bee populations have suffered high losses in much of the world  
73 (1), coincident with an increase in agricultural demand for honey bee pollination (2).  
74 Specifically, honey bee colony losses in the United States have been exacerbated  
75 since the report of Colony Collapse Disorder (CCD), a syndrome that describes  
76 large-scale, unexplained losses of managed honey bees (3-9). High levels of parasites  
77 and pathogens have been linked to the decline of honey bee colonies (10, 11).

78 *Nosema* is a genus of obligate, intracellular microsporidian parasites, which infects  
79 many diverse animal species, including honey bees (12, 13). For years, *Nosema*  
80 disease of European honey bees was exclusively attributed to a single *Nosema* species  
81 - *Nosema apis*. Another species *N. ceranae* was originally detected in Asian honey  
82 bees, *A. cerana* (14), and subsequently found to infect European honey bees, *A.*  
83 *mellifera* (15, 16). Since then, the infection of *A. mellifera* by *N. ceranae* has been  
84 reported worldwide (17-20), and nosemosis of *A. mellifera* caused by *N. ceranae* is  
85 now far more prevalent than that by its native sympatric congener *N. apis* (17, 21-25).  
86 Although there are no outward disease symptoms reported (12), *N. ceranae* infection  
87 can cause worker bee energetic stress and behavioral changes (26-29), leading to  
88 reduced lifespan of infected bees. As an emerging parasite, *N. ceranae* has often been  
89 linked to colony losses worldwide. A study based on Spanish honey bee populations  
90 showed that natural infection by *N. ceranae* could cause colony collapse (30, 31).  
91 New evidence has shown that *N. ceranae* synergistically interacts with pesticides,

92 resulting in more complex and severe diseases in honey bees (32-35). So far, the only  
93 registered treatment for nosemosis in North America is fumagillin. With prolonged  
94 use of fumagillin, the issues of drug resistance have arisen (36). As a result, novel  
95 parasite-specific and environmentally friendly therapeutic options are urgently needed  
96 for *Nosema* treatment.

97 RNA interference (RNAi), a post-transcriptional gene silencing mechanism, is an  
98 efficient and specific method of gene silencing which functions by inducing  
99 degradation of homologous mRNAs (37, 38). RNAi technology has been explored to  
100 protect honey bees from infection by pathogens and parasites (39). Three honey bee  
101 viruses, *Israeli acute paralysis virus* (IAPV), *Deformed wing virus* (DWV), and  
102 *Chinese sacbrood virus* (CSBV) have been successfully inhibited by RNAi under  
103 laboratory conditions by feeding bees with virus-specific dsRNAs/siRNAs (40-43).  
104 Moreover, a large-scale field application of IAPV dsRNA improved bee survival,  
105 colony size and honey yield (44). RNAi has also been used to help control the  
106 parasitic mite *Varroa destructor* (45). In one previous study, *N. ceranae* ADP/ATP  
107 transporter genes were targeted and silenced by corresponding dsRNAs, resulting in  
108 the decline of spore loads and alleviation of diseases in infected bees (46).

109 All honey bee disease applications of RNAi to date have targeted the genes of  
110 parasites or pathogens. Nevertheless, disease always involves interactions between  
111 hosts and parasites/pathogens, and it is also possible to mitigate infections from the  
112 host perspective, i.e. to use RNAi to manipulate host factors that interact with

113 parasites or pathogens. In fact, *Nosema* infection dramatically alters honey bee  
114 transcriptional responses (47-49), providing potential targets for host-based RNAi  
115 manipulation.

116 The Wnt signaling pathway is an important regulator of immune function in  
117 mammals (50), and recently has been found to function in regulating immune  
118 pathways, specifically Toll pathways, in insects (*Drosophila*) (51). There are several  
119 genes that serve as antagonists of the Wnt pathway and thus upregulated expression of  
120 these genes should suppress immune function. One of these genes has been found in  
121 *Drosophila* - the *naked cuticle* gene (52). As suppressors of immune function, these  
122 may serve as excellent targets for parasite manipulation of the hosts' transcriptional  
123 pathways - if they parasite can up-regulate these antagonists, it can suppress the hosts  
124 immune response. In light of the fact that *nkd* is a negative regulator of Wnt signaling  
125 pathway, we hypothesized that *nkd* expression might be regulated by *Nosema*  
126 infection and thereby could serve as a potential target of RNAi to mitigate the *Nosema*  
127 infection in honey bees. Here we confirmed *nkd* mRNA levels were upregulated when  
128 bees were infected by *N. ceranae*. Our RNAi experiments showed that silencing *nkd*  
129 led to the enhancement of immune responses through increased immune gene  
130 expression, reduction in parasite spore load, and improvement in life expectancy of *N.*  
131 *ceranae* infected bees. We provide unequivocal evidence that silencing the *nkd* gene is  
132 an efficient way to control *N. ceranae* infection and improve honey bee health.

133

134 **METHODS AND MATERIALS**

135 **Honey bees.** Honey bees used in this study were collected from colonies of *Apis*  
136 *mellifera ligustica* maintained at the USDA-ARS Bee Research Laboratory, Beltsville  
137 Maryland. Newly emerged bees were obtained by removing honeycomb frames with  
138 sealed brood from strong and healthy colonies that were identified to be  
139 *Nosema*-negative, placing the frames into mesh-walled cages individually, and then  
140 maintaining the frames in an insect growth chamber at 34±1°C, 55±5% RH overnight.  
141 Emerging adult worker bees were collected the next day (< 24 hrs). In order to make  
142 sure that the experimental bees were free of *N. ceranae* infection before proceeding to  
143 the experimental inoculation, we confirmed the negative status of *Nosema* infection  
144 using a hemocytometer and light microscopy. Briefly, 30 abdomens of newly emerged  
145 bees were dissected and ground up thoroughly in 30 ml of deionized H<sub>2</sub>O. 10 µl of the  
146 homogenate was loaded onto a hemocytometer and the presence of spores was  
147 determined under light microscopy following a previously described method (53).

148 **Inoculum preparation.** *N. ceranae* spores were purified from foragers collected  
149 outside the entrance of identified *N. ceranae* infected colonies. Midguts were pulled  
150 out and homogenized in sterile, distilled water. The purification of *Nosema* spores  
151 from the homogenate was performed as described by Fries *et al.* (2013) (54). The  
152 homogenate was filtered through a nylon mesh cloth (65 µm pore size) by  
153 centrifuging it for 5 minutes at 3,000 g. The supernatant was discarded and the pellet  
154 resuspended in sterile water and centrifuged for 10 min at 5,000 g, this step was

155 repeated twice. Finally, the pellet was resuspended in dH<sub>2</sub>O and stored at room  
156 temperature for no more than one week. The inoculum was obtained by diluting the  
157 spore solution with sucrose solution, with a final concentration of  $2.0 \times 10^7$  spores/ml  
158 in 50% (m/V) sucrose solution.

159 **Inoculation.** The newly emerged bees were collected and starved for 3 hrs in an  
160 incubator (32°C, 75% RH) before inoculation with *Nosema* spores in solution.  
161 Individual feeding was performed for each bee by holding two wings of a bee on each  
162 side with one hand and feeding the bee with 5 µl inoculum (100,000 spores) with a  
163 pipette with the other hand. 30 bees were then distributed into each cup cage, which is  
164 a plastic bee-rearing cup with a top-feeder design (55). A 3 ml syringe filled with 50%  
165 (m/V) sucrose solution was inserted in the top of the cage to feed the bees and the  
166 solution was changed every three days. A small pollen patty was supplied in the  
167 bottom of the cage for 6 days. The same number of control bees (without spore  
168 inoculation) were transferred into a cup cage as well. There were 4 replicates for each  
169 group. All cup cages were maintained in an incubator (32°C, 75% RH). Five bees  
170 were sampled from each cage at 6 days, 9 days, 12 days, 15 days, and 18 days post  
171 inoculation, respectively. The collected bee samples were immediately put into -80°C  
172 and stored until processing.

173 **Production of dsRNA.** Primers were designed from the sequence of the *A.*  
174 *mellifera nkd* gene (GenBank accession no. XM\_001120899) by using the E-RNAi  
175 web service (56), and primers for *GFP* that served as the control gene were used from



176 previous studies (57). All primer sequences were fused with the T7 promoter  
177 sequence (underlined; see Table 1). PCR reactions were performed using different  
178 templates individually: the cDNA of an adult bee was used for the amplification of  
179 *nkd*, and the pGFP vector (Clontech) was used for that of *GFP*. The 100  $\mu$ l PCR  
180 reaction mixture contained the following: 78 $\mu$ l H<sub>2</sub>O, 10 $\mu$ l 10 $\times$  reaction buffer  
181 (Invitrogen), 3 $\mu$ l MgCl<sub>2</sub>, 2 $\mu$ l dNTP mix (10 mM; Invitrogen), 2 $\mu$ l forward primer (20  
182  $\mu$ M), 2 $\mu$ l reverse primer (20  $\mu$ M), 1 $\mu$ l Taq polymerase (Invitrogen), and 2 $\mu$ l DNA  
183 template. The PCR program was 94°C for 3min, followed by 35 cycles of 94°C for  
184 30s, 56°C for 30s and 72°C for 90s, and 72°C for 10 min. After each PCR  
185 amplification, the products were then verified in 1.0% agarose gels, purified, and then  
186 used as the templates for the *in vitro* transcription reaction. The production of dsRNAs  
187 was carried out by using the MEGAscript RNAi Kit (Ambion). The transcription  
188 reactions were assembled according to the manufacturer's instruction, and the time of  
189 incubation at 37°C was extended to 15hrs. The following steps such as nuclease  
190 digestion, purification, and elution were performed using the materials associated with  
191 the kit. The quality of the dsRNAs was tested using 1.0% agarose gels and their  
192 concentration was determined with a Nanodrop 8000 spectrophotometer (Thermo  
193 Fisher Scientific Inc.). The products were diluted with sucrose solution to final  
194 concentrations of 10 $\mu$ g/ml, 20 $\mu$ g/ml, and 40 $\mu$ g/ml dsRNA in 50% (m/V) sucrose  
195 solution, respectively. The final solutions were stored at -80°C until use.

196 **RNA interference treatment.** Before the RNAi treatment, the newly-emerged bees

197 were collected and inoculated with *N. ceranae* spores as described above, and then  
198 transferred into bee rearing cages. In each cage, 20 bees were supplied with 1.5ml of  
199 50% sucrose solution containing *nkd* or *GFP* dsRNA in a 3 ml syringe and a small  
200 pollen patty in the bottom of the cage at the same day. Bees were fed with the dsRNA  
201 for 15 days; feedings were changed daily. The pollen patty was supplied for the first  
202 six days and changed every three days. All cages were incubated at 32°C, 75% RH,  
203 and the dead bees were removed every day.

204 To test the efficiency of gene knockdown, three different concentrations of *nkd*  
205 dsRNA solution, 10µg/ml, 20µg/ml, and 40µg/ml, were applied to separate cages, and  
206 20µg/ml *GFP* dsRNA solution was used for control bees. There were three replicates  
207 for each treatment. The bees were sampled at 9 days (D9) and 15 days (D15) post the  
208 ingestion of dsRNAs and stored at -80 °C until use.

209 To study the biological responses to the knockdown of *nkd*, 20µg/ml of *nkd* dsRNA  
210 and *GFP* dsRNA were fed to the infected bees. Another control group was set up with  
211 bees without any treatment and fed only 50% sucrose solution and pollen. Each group  
212 contained three replicates. The number of dead bees was recorded daily and were then  
213 removed. All the bees were collected at D15 and stored at -80 °C until use.

214 **Spore counting.** To evaluate the *Nosema* infection levels of the RNAi-treated bees,  
215 the number of spores were counted in individual bees. First, the abdomens were  
216 separated, put into 1.5ml Eppendorf tubes individually, and homogenized thoroughly  
217 in 1ml dH<sub>2</sub>O using a pestle. Then each homogenate was diluted 100 times. 10 µl of

218 the diluted solution was loaded onto a hemocytometer and the number of spores was  
219 counted under light microscopy as described by Cantwell, 1970 (53). The spore load  
220 was obtained using the following formula, (spore counts  $\times$  diluted times) / 20.

221 **RNA extraction and cDNA synthesis.** TRIzol reagent (Invitrogen) was used to  
222 extract total RNA from the abdomen of individual bees following the manufacturer's  
223 protocols. Any genomic DNA contamination was removed by treatment with DNase I  
224 (DNA-free kit, Ambion). The purity and quantity of RNA samples were examined by  
225 using a Nanodrop 8000 spectrophotometer (Thermo Fisher Scientific Inc.). All RNAs  
226 were stored at -80°C until use. First-strand cDNA was produced from a 20- $\mu$ l  
227 reverse-transcriptional reaction mixture which contained 2  $\mu$ l total RNA  
228 (approximately 1 $\mu$ g/ $\mu$ l), 1  $\mu$ l dNTP mix (10 mM), 1  $\mu$ l random primers (0.15  $\mu$ g/ $\mu$ l), 1  
229  $\mu$ l DTT (0.1 M), 4  $\mu$ l 5 $\times$ First-Strand Buffer, 1  $\mu$ l SuperScript III RT (200 U/ $\mu$ l,  
230 Invitrogen), and 10  $\mu$ l Nuclease-free water. The reaction program was as follows:  
231 25°C for 5 min, 50°C for 45 min followed by 70°C for 15 min. The cDNAs were  
232 stored at -20°C until use.

233 **Quantitative PCR.** Quantitative PCR (qPCR) was run on a CFX384 Touch  
234 Real-Time PCR System (Bio-Rad, Hercules, CA), and SYBR Green was selected as  
235 the detection signal. Primers used here were designed with Primer3 (58) (Table 1).  
236  *$\beta$ -actin* served as the reference gene and all primer pairs were validated as described  
237 by (59). Each 10  $\mu$ l PCR reaction was assembled by mixing 5  $\mu$ l 2 $\times$  Brilliant III  
238 Ultra-Fast SYBR Green QPCR Mix (Stratagene, La Jolla, CA), 0.25  $\mu$ l forward

239 primer (20 mM), 0.25  $\mu$ l reverse primer (20 mM), 0.5  $\mu$ l cDNA, and 4  $\mu$ l  
240 nuclease-free water. Each reaction was run in duplicate. The PCR program was 95°C  
241 for 3 min, followed by 40 cycles of 95°C for 10 s, 60°C for 45 s. Melting curves and  
242 no template control (NTC) reactions were monitored to evaluate the quality and  
243 specificity of amplification. Only a single peak was seen in all melting curves and no  
244 peaks showed in the NTCs. PCR products were run on a 1% agarose gel to confirm  
245 the expected amplification sizes. The threshold cycle values ( $C_t$ ) were generated by  
246 the CFX Manager 3.1 (Bio-Rad). The relative quantification of gene expression was  
247 calculated with the comparative  $C_t$  method ( $\Delta\Delta C_t$  Method) (60). For each gene, the  
248 average  $C_t$  value of the target was normalized with the corresponding  $\beta$ -actin  
249 following the formula:  $\Delta C_t = (\text{Average } C_{t_{\text{target}}}) - (\text{Average } C_{t_{\beta\text{-actin}}})$ , and the group of  
250 bees with the lowest level of gene expression was chosen as a calibrator ( $\Delta C_{t_{\text{calibrator}}}$ ).  
251 The  $\Delta C_t$  value of each group was subtracted from the  $\Delta C_{t_{\text{calibrator}}}$  to generate  $\Delta\Delta C_t$ .  
252 The concentration of each target in each group was calculated using the formula  
253  $2^{-(\Delta\Delta C_t)}$ , and expressed as an n-fold difference relative to the calibrator.

254 **Bioinformatic and statistical analyses.** Multiple alignments of insect *nkd* protein  
255 sequences was carried out with ClustalX 2.0 (61). Protein domain identification and  
256 secondary structure prediction of *A. mellifera nkd* were respectively performed with  
257 the InterProScan tool and EMBOSS: garnier algorithm of Geneious v9.1.3  
258 (Biomatters; available from [www.geneious.com](http://www.geneious.com)).

259 Dynamics of *nkd* gene expression during the course of *Nosema* infection, immune

260 gene expression, and spore load of infected bees after RNAi treatment were analyzed  
261 using independent samples *t*-tests. mRNA levels of *nkd* after dsRNA treatment were  
262 analyzed by one-way ANOVA with all compared groups passing an equal variance  
263 test, and the post-hoc effects were determined using Tukey HSD tests. Survival  
264 analysis was performed using the Kaplan-Meier method, and log-rank and Wilcoxon  
265 tests were computed to assess the overall homogeneity between the treatment strata.  
266 Pairwise comparisons were carried out using Wilcoxon tests. In all cases,  $P < 0.05$   
267 was taken to be significant. All analyses were carried out by PASW Statistics 18  
268 (SPSS Inc.).

269

## 270 **RESULTS**

271 **Identification of a highly conserved *nkd* gene in honey bees.** The identified honey  
272 bee *nkd* gene encodes a predicted protein of 545 aa, which shares 92.9% and 89.2%  
273 sequence identity with the bumble bee *Bombus impatiens* and stingless bee *Melipona*  
274 *quadrifasciata*, respectively (Fig. 1). This protein is highly conserved in Hymenoptera,  
275 as remarkable sequence identity is also seen in non-Apidae species, ranging from 65.5%  
276 (*Camponotus floridanus*) to 85.8% (*Megachile rotundata*) (Fig. 1). A conserved  
277 EF-hand domain (InterPro ID: IPR002048) - which is known to bind calcium - was  
278 identified in the *nkd* proteins (Fig. 1). Additional conserved regions included a region  
279 responsible for interaction with *Dishevelled* (*Dsh*), a component of the Wnt signaling  
280 pathway (62), and the nuclear localization motif required for nuclear localization and

281 inhibition of Wnt signaling (63) (Fig. 1). Although the diversity of *nkd* protein  
282 sequences dramatically increases when expanding the comparison to different insect  
283 orders, the sequence features mentioned above are still highly conserved. (Fig. S1)

284 ***N. ceranae* infection up-regulates the expression of *nkd* gene.** As shown in Fig. 2,  
285 the dynamics of the *nkd* gene expression was altered during the *N. ceranae* infection.  
286 The expression of *nkd* in the infected bees was significantly upregulated compared  
287 with that of control bees at Day 6 (D6) and Day 18 (D18) post inoculation (Fig. 2. for  
288 D6, *t*-test:  $t = -2.774$ ,  $df = 9$ ,  $P = 0.022$ ; for D18, *t*-test:  $t = -3.387$ ,  $df = 10.860$ ,  $P =$   
289  $0.006$ ). Collectively, *N. ceranae* infection upregulated the *nkd* gene expression (*t*-test:  
290  $t = -2.872$ ,  $df = 63$ ,  $P = 0.006$ ).

291 **Ingestion of dsRNA silences the *nkd* gene expression in *N. ceranae* infected**  
292 **bees.** As it is shown in Fig. 3, 9 days feeding of *nkd* dsRNA was insufficient to reduce  
293 the mRNA levels of the target gene in adult bees (ANOVA:  $F_{1,11} = 0.442$ ,  $P = 0.742$ ),  
294 but the gene expression can be significantly silenced by 15 days ingestion of the  
295 corresponding dsRNA (ANOVA:  $F_{1,11} = 9.1781$ ,  $P = 0.005$ ). Moreover, the  
296 knockdown of *nkd* exhibited a dosage-dependent manner (Fig. 3): along with the  
297 increase of dsRNA concentration, the effect of gene silencing also increased. When 10  
298  $\mu\text{g/ml}$  of dsRNA was applied, a 40% knockdown of *nkd* mRNA was achieved, while  
299 higher amounts of 20 or 40  $\mu\text{g/ml}$  dsRNA resulted in 50% and 70% gene silencing,  
300 and the differences compared to controls became significant (Fig. 3.  $P = 0.018$  for 20  
301  $\mu\text{g/ml}$ ;  $P = 0.004$  for 40  $\mu\text{g/ml}$ ).

302 **Silencing of the *nkd* gene up-regulates immune gene expression in *N. ceranae***  
303 **infected bees.** As shown in Fig. 4, the *nkd*-silenced bees expressed significantly  
304 higher amounts of mRNA for three antimicrobial peptide (AMP) genes *Abaecin*,  
305 *Apidaecin*, and *Defensin-1* relative to the control bees (Fig. 4. *t*-test: for *Abaecin*, *t* =  
306 -3.689, *df* = 9, *P* = 0.005; for *Apidaecin*, *t* = -3.047, *df* = 4.982, *P* = 0.029; for  
307 *Defensin-1*, *t* = -2.855, *df* = 9, *P* = 0.019). Moreover, the expression of a  
308 peptidoglycan recognition protein (PGRP) gene, *PGRP-S2* was also significantly  
309 up-regulated in *nkd*-silenced bees compared with the control bees (Fig. 4. *t*-test, *t* =  
310 -3.183, *df* = 5.043, *P* = 0.024).

311 **Silencing of the *nkd* gene reduces the *Nosema* spore levels and extends the**  
312 **lifespan of *N. ceranae* infected bees.** After feeding with *nkd* dsRNA, the spore load  
313 of infected bees was significantly (approximately 50%) lower than controls (fed with  
314 GFP dsRNA) (Fig. 5. *t*-test: *t* = 2.458, *df* = 15.485, *P* = 0.026), indicating that  
315 knockdown of the *nkd* gene expression results in a decrease in *Nosema* infection  
316 levels. Survival analysis was performed to further examine the effect of silencing the  
317 *nkd* gene on the lifespan of honey bees after *Nosema* infection. The survival  
318 distributions for the tested groups were significantly different (Fig 6. log-rank test:  $\chi^2$   
319 = 24.472, *df* = 2, *P* < 0.001; Wilcoxon test:  $\chi^2$  = 25.020, *df* = 2, *P* < 0.001). A *Nosema*  
320 infection indeed induced higher mortality in the infected honey bees (Fig 6. No  
321 treatment vs GFP-dsRNA: Wilcoxon test,  $\chi^2$  = 25.218, *P* < 0.001), however, silencing  
322 of *nkd* significantly reduced the incidence of death of adult bees (Fig 6. *nkd*-dsRNA

323 vs GFP-dsRNA: Wilcoxon test,  $\chi^2 = 4.165$ ,  $P = 0.041$ ).

324

## 325 **DISCUSSION**

326 Significant progress has been made in exploring RNAi as a therapeutic strategy for  
327 controlling diseases in honey bees, with much attention focused on pathogen-specific  
328 virulence determinants. *Nosema* are obligate intracellular parasites and therefore  
329 require host cell proteins and pathways to support their replication and many phases  
330 of their lifecycles (12, 13). In the present study, the identification and characterization  
331 of a host-based factor that is required for parasite pathogenesis in hosts allow us to  
332 obtain important insights into the host-parasite interactions and to propose a potential  
333 drug target against the parasite.

334 Wnt signaling is an evolutionarily conserved pathway that plays a critical role in  
335 embryogenesis, cell proliferation and differentiation (64). Additionally, recent studies  
336 found that activation of the Wnt signaling pathway regulates the immune response to  
337 certain pathogenic bacterial infections by up-regulating the expression of  
338 anti-inflammatory factors and down-regulating the expression of inflammatory factors  
339 (65-68). *nkd* is linked to Wnt signaling and was originally found to act as an inducible  
340 antagonist of Wnt signaling during embryonic development in *Drosophila* (52). The  
341 results of our study confirmed our hypothesis that *nkd* and Wnt signaling might be  
342 also involved in the honey bee responses to infections of *Nosema*. Indeed, *Nosema*  
343 infection can result in dramatic host transcriptional responses (47-49), and the



344 expression of *nkd* gene is significantly upregulated during the *N. ceranae* infection in  
345 honey bees, suggesting that *nkd* and Wnt signaling might be targeted by *N. ceranae*  
346 during the infection process to alter the defensive function of hosts.

347 Knockdown of *nkd* by feeding *Nosema*-infected bees with dsRNA specific to *nkd*  
348 led to several remarkable alternations, one of which was the modulation of host  
349 immune responses. Previous studies have revealed that *N. ceranae* infection induces  
350 immunosuppression in honey bees by down-regulating several immune genes such as  
351 AMP genes, *Apidaecin*, *Abaecin*, *Defensin-1*, and, *Hymenoptaecin* (69, 70). Our  
352 results show that silencing the *nkd* gene in bees infected with *N. ceranae* can reverse  
353 immune suppression and enhance the host immune response. The exact mechanism of  
354 host immune induction by *nkd* silencing remains unclear. However, silencing of the  
355 *nkd* gene ultimately reduces the *Nosema* infection levels and extends the lifespan of  
356 infected adult bees.

357 Since infection always involves the interaction between host and parasite/pathogen,  
358 infections can theoretically be controlled by targeting the host of the  
359 parasite/pathogen. All previous gene-based efforts to control honey bee parasites or  
360 pathogens have targeted these biotic threats directly. Here, by targeting the honey bee  
361 *nkd* gene with RNAi, we demonstrate that silencing a honey bee gene can suppress  
362 the reproduction of parasites/pathogens and improve the overall health of honey bees.  
363 Our results provide a novel host-derived strategy to mitigate honey bee disease.  
364 Similar studies have been reported in various species. For example, silencing of the

365 *Cactus* gene, an inhibitor of Toll pathway, reduces the extent of dengue virus infection  
366 in the midgut by 4 fold in the mosquitos, *Aedes aegypti* (71). Down-regulation of  
367 *scavenger receptor class B type 1 (SR-B1)* expression by RNAi dramatically  
368 decreases the susceptibility of human hepatoma cells to Hepatitis C virus (HCC)  
369 infection, resulting in the inhibition of this virus infection (72). These studies together  
370 indicate that targeting host factors by RNAi can potentially prevent the hosts from  
371 infections of parasites/pathogens and promote the overall health of hosts.

372 RNAi technology has great potential for relieving the impacts of honey bee  
373 diseases. Other previous studies (46), combined with our efforts, demonstrate that  
374 silencing the parasite/host genes by RNAi manipulation is efficient to suppress  
375 parasite development and improve honey bee health to some extent in the laboratory.  
376 It is likely that the combination of both strategies, meaning targeting both host and  
377 parasite genes in the same RNAi manipulation, will lead to better results for  
378 controlling *Nosema* infection. As for field application, more experiments are needed  
379 to determine ideal treatment time, the suitable concentration of dsRNA, and other  
380 factors. This present study will help direct the application of RNAi to mitigate *N.*  
381 *ceranae* infection in honey bees.

382 In sum, these studies have identified a host factor required for *Nosema* infection  
383 and highlight the potency of host-derived RNAi-based therapeutics to inhibit not only  
384 microsporidian parasite infection but also potentially a wide range of pathogens and  
385 parasites that cause serious diseases in honey bees.

386

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397 **REFERENCES**

- 398 1. **vanEngelsdorp D, Meixner MD.** 2010. A historical review of managed honey  
399 bee populations in Europe and the United States and the factors that may affect  
400 them. *J Invertebr Pathol* **103, Supplement:S80-S95.**
- 401 2. **Breeze TD, Vaissière BE, Bommarco R, Petanidou T, Seraphides N, Kozák L,**  
402 **Scheper J, Biesmeijer JC, Kleijn D, Gyldenkerne S, Moretti M, Holzschuh A,**  
403 **Steffan-Dewenter I, Stout JC, Pärtel M, Zobel M, Potts SG.** 2014.  
404 Agricultural policies exacerbate honeybee pollination service supply-demand  
405 mismatches across Europe. *PLoS ONE* **9:e82996.**
- 406 3. **vanEngelsdorp D, Hayes J, Underwood RM, Pettis JS.** 2010. A survey of  
407 honey bee colony losses in the United States, fall 2008 to spring 2009. *J Apic Res*  
408 **49:7-14.**
- 409 4. **vanEngelsdorp D, Hayes J, Underwood RM, Caron D, Pettis J.** 2011. A  
410 survey of managed honey bee colony losses in the USA, fall 2009 to winter 2010.  
411 *J Apic Res* **50:1-10.**
- 412 5. **vanEngelsdorp D, Caron D, Hayes J, Underwood R, Henson M, Rennich K,**  
413 **Spleen A, Andree M, Snyder R, Lee K, Roccasecca K, Wilson M, Wilkes J,**  
414 **Lengerich E, Pettis J.** 2012. A national survey of managed honey bee 2010–11  
415 winter colony losses in the USA: results from the Bee Informed Partnership. *J*  
416 *Apic Res* **51:115-124.**
- 417 6. **Spleen AM, Lengerich EJ, Rennich K, Caron D, Rose R, Pettis JS, Henson**

- 418 **M, Wilkes JT, Wilson M, Stitzinger J, Lee K, Andree M, Snyder R,**  
419 **vanEngelsdorp D.** 2013. A national survey of managed honey bee 2011–12  
420 winter colony losses in the United States: results from the Bee Informed  
421 Partnership. *J Apic Res* **52**:44-53.
- 422 7. **Steinhauer NA, Rennich K, Wilson ME, Caron DM, Lengerich EJ, Pettis JS,**  
423 **Rose R, Skinner JA, Tarpy DR, Wilkes JT, vanEngelsdorp D.** 2014. A  
424 national survey of managed honey bee 2012–2013 annual colony losses in the  
425 USA: results from the Bee Informed Partnership. *J Apic Res* **53**:1-18.
- 426 8. **vanEngelsdorp D, Hayes J, Jr., Underwood RM, Pettis J.** 2008. A survey of  
427 honey bee colony losses in the U.S., fall 2007 to spring 2008. *PLoS ONE*  
428 **3**:e4071.
- 429 9. **vanEngelsdorp D, Underwood R, Caron D, Hayes JJ.** 2007. An estimate of  
430 managed colony losses in the winter of 2006–2007: a report commissioned by the  
431 Apiary Inspectors of America. *Am Bee J* **147**:599-603.
- 432 10. **Genersch E.** 2010. Honey bee pathology: Current threats to honey bees and  
433 beekeeping. *Appl Microbiol Biotechnol* **87**:87-97.
- 434 11. **Ratnieks FLW, Carreck NL.** 2010. Clarity on Honey Bee Collapse? *Science*  
435 **327**:152-153.
- 436 12. **Fries I.** 2010. *Nosema ceranae* in European honey bees (*Apis mellifera*). *J*  
437 *Invertebr Pathol* **103, Supplement**:S73-S79.
- 438 13. **Holt HL, Grozinger CM.** 2016. Approaches and challenges to managing

- 439 *Nosema* (Microspora: Nosematidae) parasites in honey bee (Hymenoptera:  
440 Apidae) colonies. J Econ Entomol **109**:1487-1503.
- 441 14. **Fries I, Feng F, da Silva A, Slemenda SB, Pieniazek NJ.** 1996. *Nosema*  
442 *ceranae* n. sp. (Microspora, Nosematidae), morphological and molecular  
443 characterization of a microsporidian parasite of the Asian honey bee *Apis cerana*  
444 (Hymenoptera, Apidae). Eur J Protistol **32**:356-365.
- 445 15. **Higes M, Martín R, Meana A.** 2006. *Nosema ceranae*, a new microsporidian  
446 parasite in honeybees in Europe. J Invertebr Pathol **92**:93-95.
- 447 16. **Huang W-F, Jiang J-H, Chen Y-W, Wang C-H.** 2007. A *Nosema ceranae*  
448 isolate from the honeybee *Apis mellifera*. Apidologie **38**:30-37.
- 449 17. **Chen Y, Evans JD, Smith IB, Pettis JS.** 2008. *Nosema ceranae* is a long-present  
450 and wide-spread microsporidian infection of the European honey bee (*Apis*  
451 *mellifera*) in the United States. J Invertebr Pathol **97**:186-188.
- 452 18. **Giersch T, Berg T, Galea F, Hornitzky M.** 2009. *Nosema ceranae* infects honey  
453 bees (*Apis mellifera*) and contaminates honey in Australia. Apidologie  
454 **40**:117-123.
- 455 19. **Higes M, Martín-Hernández R, Garrido-Bailón E, Botías C, Meana A.** 2009.  
456 The presence of *Nosema ceranae* (Microsporidia) in North African honey bees  
457 (*Apis mellifera intermissa*). J Apic Res **48**:217-219.
- 458 20. **Invernizzi C, Abud C, Tomasco IH, Harriet J, Ramallo G, Campá J, Katz H,**  
459 **Gardiol G, Mendoza Y.** 2009. Presence of *Nosema ceranae* in honeybees (*Apis*

- 460 *mellifera*) in Uruguay. J Invertebr Pathol **101**:150-153.
- 461 21. **Martín-Hernández R, Meana A, Prieto L, Salvador AM, Garrido-Bailón E,**  
462 **Higes M.** 2007. Outcome of colonization of *Apis mellifera* by *Nosema ceranae*.  
463 Appl Environ Microbiol **73**:6331-6338.
- 464 22. **Williams GR, Shafer ABA, Rogers REL, Shutler D, Stewart DT.** 2008. First  
465 detection of *Nosema ceranae*, a microsporidian parasite of European honey bees  
466 (*Apis mellifera*), in Canada and central USA. J Invertebr Pathol **97**:189-192.
- 467 23. **Traver BE, Fell RD.** 2011. Prevalence and infection intensity of *Nosema* in  
468 honey bee (*Apis mellifera* L.) colonies in Virginia. J Invertebr Pathol **107**:43-49.
- 469 24. **Yoshiyama M, Kimura K.** 2011. Distribution of *Nosema ceranae* in the  
470 European honeybee, *Apis mellifera* in Japan. J Invertebr Pathol **106**:263-267.
- 471 25. **Martínez J, Leal G, Conget P.** 2012. *Nosema ceranae* an emergent pathogen of  
472 *Apis mellifera* in Chile. Parasitol Res **111**:601-607.
- 473 26. **Goblirsch M, Huang ZY, Spivak M.** 2013. Physiological and behavioral  
474 changes in honey bees (*Apis mellifera*) induced by *Nosema ceranae* infection.  
475 PLoS ONE **8**:e58165.
- 476 27. **Higes M, García-Palencia P, Martín-Hernández R, Meana A.** 2007.  
477 Experimental infection of *Apis mellifera* honeybees with *Nosema ceranae*  
478 (Microsporidia). J Invertebr Pathol **94**:211-217.
- 479 28. **Mayack C, Naug D.** 2009. Energetic stress in the honeybee *Apis mellifera* from  
480 *Nosema ceranae* infection. J Invertebr Pathol **100**:185-188.

- 481 29. **Paxton RJ, Klee J, Korpela S, Fries I.** 2007. *Nosema ceranae* has infected *Apis*  
482 *mellifera* in Europe since at least 1998 and may be more virulent than *Nosema*  
483 *apis*. *Apidologie* **38**:558-565.
- 484 30. **Higes M, Martín-Hernández R, Botías C, Bailón EG, González-Porto AV,**  
485 **Barrios L, del Nozal MJ, Bernal JL, Jiménez JJ, Palencia PG, Meana A.**  
486 2008. How natural infection by *Nosema ceranae* causes honeybee colony  
487 collapse. *Environ Microbiol* **10**:2659-2669.
- 488 31. **Higes M, Martín-Hernández R, Garrido-Bailón E, González-Porto AV,**  
489 **García-Palencia P, Meana A, Del Nozal MJ, Mayo R, Bernal JL.** 2009.  
490 Honeybee colony collapse due to *Nosema ceranae* in professional apiaries.  
491 *Environ Microbiol Rep* **1**:110-113.
- 492 32. **Alaux C, Brunet J-L, Dussaubat C, Mondet F, Tchamitchan S, Cousin M,**  
493 **Brillard J, Baldy A, Belzunces LP, Le Conte Y.** 2010. Interactions between  
494 *Nosema* microspores and a neonicotinoid weaken honeybees (*Apis mellifera*).  
495 *Environ Microbiol* **12**:774-782.
- 496 33. **Pettis J, vanEngelsdorp D, Johnson J, Dively G.** 2012. Pesticide exposure in  
497 honey bees results in increased levels of the gut pathogen *Nosema*.  
498 *Naturwissenschaften* **99**:153-158.
- 499 34. **Vidau C, Diogon M, Aufauvre J, Fontbonne R, Viguès B, Brunet J-L, Texier**  
500 **C, Biron DG, Blot N, El Alaoui H, Belzunces LP, Delbac F.** 2011. Exposure to  
501 sublethal doses of fipronil and thiacloprid highly increases mortality of



502 honeybees previously infected by *Nosema ceranae*. PLoS ONE **6**:e21550.

503 35. **Wu JY, Smart MD, Anelli CM, Sheppard WS.** 2012. Honey bees (*Apis*  
504 *mellifera*) reared in brood combs containing high levels of pesticide residues  
505 exhibit increased susceptibility to *Nosema* (Microsporidia) infection. J Invertebr  
506 Pathol **109**:326-329.

507 36. **Huang W-F, Solter LF, Yau PM, Imai BS.** 2013. *Nosema ceranae* escapes  
508 fumagillin control in honey bees. PLoS Path **9**:e1003185.

509 37. **Hannon GJ.** 2002. RNA interference. Nature **418**:244-251.

510 38. **Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC.** 1998.  
511 Potent and specific genetic interference by double-stranded RNA in  
512 *Caenorhabditis elegans*. Nature **391**:806-811.

513 39. **Grozinger CM, Robinson GE.** 2015. The power and promise of applying  
514 genomics to honey bee health. Curr Opin Insect Sci **10**:124-132.

515 40. **Liu X, Zhang Y, Yan X, Han R.** 2010. Prevention of Chinese sacbrood virus  
516 infection in *Apis cerana* using RNA Interference. Curr Microbiol **61**:422-428.

517 41. **Chen YP, Pettis JS, Corona M, Chen WP, Li CJ, Spivak M, Visscher PK,**  
518 **DeGrandi-Hoffman G, Boncristiani H, Zhao Y, vanEngelsdorp D, Delaplane**  
519 **K, Solter L, Drummond F, Kramer M, Lipkin WI, Palacios G, Hamilton MC,**  
520 **Smith B, Huang SK, Zheng HQ, Li JL, Zhang X, Zhou AF, Wu LY, Zhou JZ,**  
521 **Lee M-L, Teixeira EW, Li ZG, Evans JD.** 2014. Israeli acute paralysis virus:  
522 epidemiology, pathogenesis and implications for honey bee health. PLoS Path

- 523        **10:e1004261.**
- 524    42. **Desai SD, Eu YJ, Whyard S, Currie RW.** 2012. Reduction in deformed wing  
525        virus infection in larval and adult honey bees (*Apis mellifera* L.) by  
526        double-stranded RNA ingestion. *Insect Mol Biol* **21**:446-455.
- 527    43. **Maori E, Paldi N, Shafir S, Kalev H, Tsur E, Glick E, Sela I.** 2009. IAPV, a  
528        bee-affecting virus associated with Colony Collapse Disorder can be silenced by  
529        dsRNA ingestion. *Insect Mol Biol* **18**:55-60.
- 530    44. **Hunter W, Ellis J, vanEngelsdorp D, Hayes J, Westervelt D, Glick E,**  
531        **Williams M, Sela I, Maori E, Pettis J, Cox-Foster D, Paldi N.** 2010.  
532        Large-scale field application of RNAi technology reducing Israeli acute paralysis  
533        virus disease in honey bees (*Apis mellifera*, Hymenoptera: Apidae). *PLoS Path*  
534        **6**:e1001160.
- 535    45. **Garbian Y, Maori E, Kalev H, Shafir S, Sela I.** 2012. Bidirectional transfer of  
536        RNAi between honey bee and *Varroa destructor*: *Varroa* gene silencing reduces  
537        *Varroa* population. *PLoS Path* **8**:e1003035.
- 538    46. **Paldi N, Glick E, Oliva M, Zilberberg Y, Aubin L, Pettis J, Chen Y, Evans JD.**  
539        2010. Effective gene silencing in a microsporidian parasite associated with  
540        honeybee (*Apis mellifera*) colony declines. *Appl Environ Microbiol*  
541        **76**:5960-5964.
- 542    47. **Aufauvre J, Misme-Aucouturier B, Viguès B, Texier C, Delbac F, Blot N.**  
543        2014. Transcriptome analyses of the honeybee response to *Nosema ceranae* and

- 544 insecticides. PLoS ONE **9**:e91686.
- 545 48. **Dussaubat C, Brunet J-L, Higes M, Colbourne JK, Lopez J, Choi J-H,**  
546 **Martín-Hernández R, Botías C, Cousin M, McDonnell C, Bonnet M,**  
547 **Belzunces LP, Moritz RFA, Le Conte Y, Alaux C.** 2012. Gut pathology and  
548 responses to the microsporidium *Nosema ceranae* in the honey bee *Apis mellifera*.  
549 PLoS ONE **7**:e37017.
- 550 49. **Holt H, Aronstein K, Grozinger C.** 2013. Chronic parasitization by *Nosema*  
551 microsporidia causes global expression changes in core nutritional, metabolic and  
552 behavioral pathways in honey bee workers (*Apis mellifera*). BMC Genomics  
553 **14**:799.
- 554 50. **Staal FJT, Luis TC, Tiemessen MM.** 2008. WNT signalling in the immune  
555 system: WNT is spreading its wings. Nat Rev Immunol **8**:581-593.
- 556 51. **Gordon MD, Dionne MS, Schneider DS, Nusse R.** 2005. WntD is a feedback  
557 inhibitor of Dorsal/NF- $\kappa$ B in *Drosophila* development and immunity.  
558 Nature **437**:746-749.
- 559 52. **Zeng W, Wharton KA, Mack JA, Wang K, Gadbaw M, Suyama K, Klein PS,**  
560 **Scott MP.** 2000. *naked cuticle* encodes an inducible antagonist of Wnt signalling.  
561 Nature **403**:789-795.
- 562 53. **Cantwell GE.** 1970. Standard methods for counting *Nosema* spores. Am Bee J  
563 **110**:222-223.
- 564 54. **Fries I, Chauzat MP, Chen YP, Doublet V, Genersch E, Gisder S, Higes M,**

- 565 **McMahon DP, Martin-Hernandez R, Natsopoulou M, Paxton RJ, Tanner G,**  
566 **Webster TC, Williams GR.** 2013. Standard methods for *Nosema* research. J  
567 Apic Res **52**.
- 568 55. **Evans JD, Chen YP, Prisco Gd, Pettis J, Williams V.** 2009. Bee cups:  
569 single-use cages for honey bee experiments. J Apic Res **48**:300-302.
- 570 56. **Horn T, Boutros M.** 2010. E-RNAi: a web application for the multi-species  
571 design of RNAi reagents—2010 update. Nucleic Acids Res **38**:W332-W339.
- 572 57. **Amdam GV, Norberg K, Page JRE, Erber J, Scheiner R.** 2006.  
573 Downregulation of *vitellogenin* gene activity increases the gustatory  
574 responsiveness of honey bee workers (*Apis mellifera*). Behav Brain Res  
575 **169**:201-205.
- 576 58. **Untergasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M,**  
577 **Rozen SG.** 2012. Primer3—new capabilities and interfaces. Nucleic Acids Res  
578 **40**:e115.
- 579 59. **Li W, Huang ZY, Liu F, Li Z, Yan L, Zhang S, Chen S, Zhong B, Su S.** 2013.  
580 Molecular Cloning and characterization of juvenile hormone acid  
581 methyltransferase in the honey bee, *Apis mellifera*, and its differential expression  
582 during caste differentiation. PLoS ONE **8**:e68544.
- 583 60. **Schmittgen TD, Livak KJ.** 2008. Analyzing real-time PCR data by the  
584 comparative CT method. Nature Protocols **3**:1101-1108.
- 585 61. **Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA,**

- 586 **McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD,**  
587 **Gibson TJ, Higgins DG.** 2007. Clustal W and Clustal X version 2.0.  
588 Bioinformatics **23**:2947-2948.
- 589 62. **Rousset R, Mack JA, Wharton KA, Axelrod JD, Cadigan KM, Fish MP,**  
590 **Nusse R, Scott MP.** 2001. *naked cuticle* targets *dishevelled* to antagonize Wnt  
591 signal transduction. Genes Dev **15**:658-671.
- 592 63. **Waldrop S, Chan C-C, Cagatay T, Zhang S, Rousset R, Mack J, Zeng W,**  
593 **Fish M, Zhang M, Amanai M, Wharton KA.** 2006. An unconventional nuclear  
594 localization motif is crucial for function of the *Drosophila* Wnt/Wingless  
595 antagonist *naked cuticle*. Genetics **174**:331-348.
- 596 64. **Logan CY, Nusse R.** 2004. The Wnt signaling pathway in development and  
597 disease. Annu Rev Cell Dev Biol **20**:781-810.
- 598 65. **Chen K, Yin L, Nie X, Deng Q, Wu Y, Zhu M, Li D, Li M, Wu M, Huang X.**  
599 2013.  $\beta$ -Catenin promotes host resistance against *Pseudomonas aeruginosa*  
600 keratitis. J Infect **67**:584-594.
- 601 66. **Liu X, Wu S, Xia Y, Li XE, Xia Y, Zhou ZD, Sun J.** 2011. Wingless homolog  
602 Wnt11 suppresses bacterial invasion and inflammation in intestinal epithelial  
603 cells. Am J Physiol-gastr L **301**:G992-G1003.
- 604 67. **Silva-García O, Valdez-Alarcón JJ, Baizabal-Aguirre VM.** 2014. The  
605 Wnt/ $\beta$ -catenin signaling pathway controls the inflammatory response in  
606 infections caused by pathogenic bacteria. Mediat Inflamm **2014**:7.

- 607 68. **Liu X, Lu R, Wu S, Zhang Y-g, Xia Y, Sartor RB, Sun J.** 2012. Wnt2 inhibits  
608 enteric bacterial-induced inflammation in intestinal epithelial cells. *Inflamm*  
609 *Bowel Dis* **18**:418-429.
- 610 69. **Antúnez K, Martín-Hernández R, Prieto L, Meana A, Zunino P, Higes M.**  
611 2009. Immune suppression in the honey bee (*Apis mellifera*) following infection  
612 by *Nosema ceranae* (Microsporidia). *Environ Microbiol* **11**:2284-2290.
- 613 70. **Chaimanee V, Chantawannakul P, Chen Y, Evans JD, Pettis JS.** 2012.  
614 Differential expression of immune genes of adult honey bee (*Apis mellifera*) after  
615 inoculated by *Nosema ceranae*. *J Insect Physiol* **58**:1090-1095.
- 616 71. **Xi Z, Ramirez JL, Dimopoulos G.** 2008. The *Aedes aegypti* Toll pathway  
617 controls dengue virus infection. *PLoS Pathog* **4**:e1000098.
- 618 72. **Zeisel MB, Koutsoudakis G, Schnober EK, Haberstroh A, Blum HE, Cosset**  
619 **F-L, Wakita T, Jaeck D, Doffoel M, Royer C, Soulier E, Schvoerer E,**  
620 **Schuster C, Stoll-Keller F, Bartenschlager R, Pietschmann T, Barth H,**  
621 **Baumert TF.** 2007. Scavenger receptor class B type I is a key host factor for  
622 hepatitis C virus infection required for an entry step closely linked to CD81.  
623 *Hepatology* **46**:1722-1731.
- 624 73. **Mutti NS, Dolezal AG, Wolschin F, Mutti JS, Gill KS, Amdam GV.** 2011. IRS  
625 and TOR nutrient-signaling pathways act via juvenile hormone to influence  
626 honey bee caste fate. *J Exp Biol* **214**:3977-3984.
- 627 74. **Yang X, Cox-Foster DL.** 2005. Impact of an ectoparasite on the immunity and

628 pathology of an invertebrate: Evidence for host immunosuppression and viral  
629 amplification. Proc Natl Acad Sci USA **102**:7470-7475.  
630

## 631 TABLES

632 TABLE 1 Primer sequences used in this study.

	Gene (accession no.)	Primer	Sequence (5'→3')	Amplicon location	Amplicon length (bp)	Reference
RNAi	<i>nkd</i> (XM_001120899)	nkd-RNAi-F	<u>taatacgactcactatagggcga</u> CGC GCTTATGTTCAACCTC	1889-2122	234	This study
		nkd-RNAi-R	<u>taatacgactcactatagggcga</u> GGTC GCGTGTTTCAAATGAT			
	<i>GFP</i> (AF324407)	GFP- RNAi -F	<u>taatacgactcactatagggcga</u> TTCC ATGGCCAACACTTGTCA	173-674	502	(57)
		GFP- RNAi-R	<u>taatacgactcactatagggcga</u> TCAA GAAGGACCATGTGGTC			
qPCR	<i>nkd</i>	nkd-F	AGGATGACGGTGAAAAT GCG	1365-1540	176	This study
		nkd-R	ATTAGTCGTGAGGAGAGG CG			
	<i>β-actin</i> (NM_001185145)	actin-F	TGCCAACACTGTCCTTTC TG	1018-1173	156	(73)
		actin-R	AGAATTGACCCACCAATC CA			
	<i>Abaecin</i> (NM_001011617)	Abaecin-F	AGATCTGCACACTCGAGG TCTG	14-214	201	(74)
		Abaecin-R	TCGGATTGAATGGTCCCT GA			
	<i>Apidaecin</i> (NM_001011613)	Apidaecin-F	TTTTGCCTTAGCAATTCTT GTTG	58-137	80	This study
		Apidaecin-R	GCAGGTCGAGTAGGCGG ATCT			
	<i>Defensin-1</i> (NM_001011616)	Defensin-1-F	TGTCGGCCTTCTCTTCAT GG	88-288	201	This study
		Defensin-1-R	TGACCTCCAGCTTTACCC AAA			
	<i>PGRP-S2</i> (NM_001163716)	PGRP-S2-F	TTGCACAAAATCCTCCGC C	146-274	129	This study
		PGRP-S2-R	CACCCCAACCCTTCTCAT CT			

633

634



635 **FIGURE LEGENDS**

636 **FIG 1** Sequence conservation and predicted secondary structure of *A. mellifera nkd*.

637 (A) multiple alignment of *A. mellifera* (XP\_001120899), *Bombus impatiens*

638 (XP\_012249347), *Megachile rotundata* (XP\_003702467), *Melipona quadrifasciata*

639 (KOX70301), *Atta colombica* (KYM87061), *Harpegnathos saltator* (XP\_011151892),

640 and *Camponotus floridanus* (EFN66676) *nkd* protein sequences. Black and grey

641 shadings indicate identity and high conservation of amino acids, respectively. The

642 region responsible for interaction with *dsh* is indicated with the red bar. The nuclear

643 localization motif is underlined by the green bar. The blue box highlights the EF-hand

644 domain. (B) protein domain identification and secondary structure prediction of *A.*

645 *mellifera nkd*. The conserved domain is shown with a black bar.

646

647 **FIG 2** The expression profile of *nkd* during *N. ceranae* infection. The X axis indicates

648 the days post inoculation of *Nosema* spores. The relative gene expression levels (Y

649 axis) are expressed with mean  $\pm$  SEM. The sample size is shown in the bottom of each

650 bar, and the solid five-pointed star indicates the calibrator used to normalize the gene

651 expression. Data were analyzed by independent samples *t*-test. Significant differences

652 between groups are indicated by asterisks (\*  $P < 0.05$ , \*\*  $P < 0.01$ ).

653

654 **FIG 3** Knockdown of *nkd* gene in adult bees by dsRNA ingestion. All groups of adult

655 bees were inoculated with *Nosema* spores first and then fed with sucrose solution

656 containing dsRNA for 15 days. The silencing effect was examined after 9 days (A)  
657 and 15 days (B) feeding of dsRNA, respectively. The control bees (GFP-dsRNA)  
658 were fed with the dsRNA derived from GFP sequence. For the treatment groups, three  
659 different concentration gradients of *nkd* dsRNA were examined. They were as follows,  
660 40  $\mu\text{g/ml}$  (*nkd*-dsRNA-40), 20  $\mu\text{g/ml}$  (*nkd*-dsRNA-20), and 10  $\mu\text{g/ml}$   
661 (*nkd*-dsRNA-10). The relative gene expression values are shown as mean  $\pm$  SEM. All  
662 groups are with the same sample size ( $n = 3$ ). The calibrator used to normalize the  
663 gene expression is indicated with the solid five-pointed star inside the bar. One-way  
664 ANOVA was employed to analyze the differences of data, and post-hoc effects were  
665 identified by Tukey HSD tests. Significant differences between groups are indicated  
666 by asterisks (\*  $P < 0.05$ , \*\*  $P < 0.01$ ).

667

668 **FIG 4** Effect of *nkd* gene silencing on the immune gene expression in *Nosema*  
669 infected bees. The relative mRNA levels of immune genes (X axis) were compared  
670 between control bees fed with 20  $\mu\text{g/ml}$  GFP dsRNA (GFP-dsRNA,  $n = 6$ ) and the  
671 treatment bees fed with 20  $\mu\text{g/ml}$  *nkd* dsRNA (*nkd*-dsRNA,  $n = 5$ ). Both groups were  
672 inoculated with *Nosema* spores before dsRNA feeding. The expression values are  
673 expressed with mean  $\pm$  SEM. Data were analyzed by independent samples *t*-test.  
674 Significant differences between groups are indicated by asterisks (\*  $P < 0.05$ , \*\*  $P <$   
675 0.01).

676

677 **FIG 5** Effect of *nkd* gene silencing on the *Nosema* infection levels in adult bees. The  
678 *Nosema* infection levels were determined by spore counting. Spore loads of the two  
679 groups of bees were compared, the control bees fed with 20 µg/ml GFP dsRNA ( $n =$   
680 11), and the treatment bees fed with 20 µg/ml *nkd* dsRNA ( $n = 8$ ). The values of spore  
681 load are expressed with mean  $\pm$  SEM. Significant differences of data were analyzed  
682 by independent samples *t*-test, and indicated with asterisks (\*  $P < 0.05$ ).

683

684 **FIG 6** Effect of *nkd* gene silencing on the lifespan of honey bees infected by *N.*  
685 *ceranae*. Survival curves for bees inoculated with 100,000 *N. ceranae* spores at day 0  
686 (i.e., within 24 h after adult emergence) and fed with 50% (m/V) sucrose solution  
687 containing 20 µg/ml *nkd* dsRNA (*nkd*-dsRNA,  $n = 60$ ) or 20 µg/ml GFP dsRNA  
688 (GFP-dsRNA,  $n = 60$ ) for 15 days, and for the bees without any treatment and only  
689 fed with 50% sucrose solution (No treatment,  $n = 60$ ). Knockdown of *nkd* gene  
690 reduced the incidence of death ( $P = 0.041$  by Wilcoxon test).

691

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