Impact of Nosema ceranae infection on sucrose solution consumption, midgut epithelial cell structure, and lifespan of Apis cerana cerana workers

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Abstract

Apis cerana is the original host for *N. ceranae*, and the impact of *Nosema ceranae* infection on *A. cerana* is largely unknown. In this study, workers of *Apis cerana cerana* were inoculated with *N. ceranae* spores. and the effects on the microsporidian spore load, host sucrose solution consumption, midgut epithelial cell structure, and lifespan were investigated. Spore counting suggested that the spore load in the host midguts decreased from 1 to 2 days post inoculation (dpi), while it increased from 2 to 13 dpi. No statistically significant difference in workers' sucrose solution consumption was detected between *N. ceranae*-inoculated and un-inoculated groups. Microscopic observation showed darkly stained microsporidia in the midgut epithelial cells of *N. ceranae*-inoculated workers from 7 to 10 dpi. Additionally, the boundary of *N. ceranae*-inoculated host epithelial cells was blurred, the nucleus had almost disappeared, and the nucleic acid substance was diffused, whereas the boundary of un-inoculated midgut epithelial cells remained intact and the darkly stained nucleus was clear. The survival rates of workers in both *N. ceranae*-inoculated groups and un-inoculated groups started to decrease at 5 dpi. Additionally, the survival rate of workers in *N. ceranae*-inoculated groups was nearly always lower than that in un-inoculated groups, and there was a significant difference between these two groups from 11 to 20 dpi. These results demonstrate that the spore load of *N. ceranae* continuously elevated with microsporidian proliferation, causing damage to the midgut epithelial cell structure and shortening the host's lifespan. Our findings offer a solid basis for exploring the molecular mechanism underlying *N. ceranae* infection and *N. ceranae*– *A. cerana* interaction.

Introduction

Apis cerana is the original host for *Nosema ceranae*, a fungal parasite that causes bee nosemosis, a chronic disease that frequently occurs in colonies throughout the world (Klee et al. 2007; Pașca et al. 2021). After long-term interactions and co-evolution, *A. cerana* and *N. ceranae* have adapted to living with each other (Li et al. 2012). Since the first identification of *N. ceranae* in *A. ceranae* by Fries *et al.* (Fries et al. 1996), N. *ceranae* has been reported to infect European honeybees (*Apis mellifera*) in Taiwan, Europe, North America (Klee et al. 2007; Huang et al. 2007; Higes et al. 2006). Currently, *N. ceranae* is the most predominant microsporidian in honeybees worldwide (Martín-Hernández et al. 2018).

Previous studies suggested that *N. ceranae* infection could lead to damage to the midgut epithelial cell structure, an increase in the consumption of sucrose solution, inhibition of apoptosis and immunosuppression, earlier foraging activity, as well as a shortening of the lifespan for European honeybee (*Apis mellifera*) workers (García-Palencia et al. 2010; Mayack and Naug 2009; Kurze et al, 2018; Goblirsch et al. 2013). However, few studies have investigated the influence of *N. ceranae* infection on *A. cerana* (Wu et al. 2020; Sinpoo et al. 2018), and a deeper understanding of microsporidian infection and microsporidian–host interaction is lacking.

Apis cerana cerana, the nominated *A. cerana* species, is a bee species that exists in China and is widely used in beekeeping practice in many Asian countries (Lin et al. 2016). To decipher the interaction between

N. ceranae and *Apis cerana cerana* workers at the molecular level, our team previously identified the miRNA response and miRNA-regulated network of the host following *N. ceranae* infection (Chen et al. 2019) and performed a comprehensive investigation of the profiles of highly expressed genes in both the host and microsporidian (Fu et al. 2020; Xiong et al. 2020). Recently, our group investigated the immune response of *A. c. cerana* workers to *N. ceranae* infection using a transcriptomic investigation and revealed that different cellular and humoral immune responses were utilized by *A. c. cerana* and *Apis mellifera ligustica* workers to defend themselves against infection by *N. ceranae* (Xing et al. 2021).

At present, the influence of *N. ceranae* infection on *A. cerana* is largely unknown. In the present study, to evaluate the impact of *N. ceranae* infection on *A. c. cerana* workers, *N. ceranae* spores were purified and used to inoculate newly emerged *A. c. cerana* workers. This was followed by an in-depth investigation of the microsporidian spore load as well as the host sucrose solution consumption and survival rate. Additionally, paraffin sections of host midgut tissue were prepared and subjected to microscopic observation. Our data not only offer valuable experimental evidence for *N. ceranae* infection of *A. c. cerana* workers but also contribute to the dissection of the host response, microsporidian infection, and host–microsporidian interaction.

Materials And Methods

Collection of bees for spore extraction and spore inoculation

A. c. cerana workers were obtained from colonies in the teaching apiary of the College of Animal Sciences (College of Bee Science), Fujian Agricultural and Forestry University. Microscopic observation and PCR detection showed that these workers were *Nosema*-free. No Varroa was observed during the experiment, and there were no specific bands amplified from *N. apis*, *N. ceranae*, or several viruses, such as SBV, DWV, IAPV, BQCV, KBV, and CBPV, on the basis of RT-PCR with specific corresponding primers (Xing et al. 2021).

N. ceranae-infected *A. c. cerana* workers were obtained from an apiary located in Minhou County, Fuzhou City, China. *N. ceranae* spores had previously been prepared using a discontinuous density gradient Percoll method (Xing et al. 2021). Briefly, a total of 200 foragers were collected from an infected colony. The midguts were dissected and then homogenized in distilled water, followed by filtration through four layers of sterile gauze and three cycles of centrifugation at 6000× g for 5 min. The sediment was resuspended and purified with a discontinuous Percoll gradient (Solarbio). The spore pellet was isolated using a sterile syringe followed by centrifugation on a discontinuous Percoll gradient.

Experimental inoculation and artificial rearing of workers

Before artificial inoculation, the prepared microsporidian spores were observed microscopically (Fig. 1A) and examined by PCR using specific primers for *N. ceranae* (F: CGGATAAAAGAGTCCGTTACC; R: TGAGCAGGGTTCTAGGGAT), as previously described by Chen *et al* (Chen et al. 2019) (Fig. 1B). Inoculation and rearing of *A. c. cerana* workers were performed following our previously established

protocol (Chen et al. 2019) (Fig. 1C-E). In this study, at 24 h after emergence, members of the treatment group (n = 35) were each fed 5 μL of 50% (w/v) sucrose solution containing *N. ceranae* spores (1×10^6 per mL), while members of the control group (n = 35) were fed 5 μL of sucrose solution without microsporidian spores. The treatment group (or control group) contained six plastic cages. All workers were reared in an incubator at 34 ± 0.5 ℃ and 60–70% RH. After 24 h, the honeybees were fed ad libitum with a feeder containing 4 mL of 50% (w/v) sucrose solution, and the feeders were replaced daily throughout the experiment. Each cage was carefully checked, and the dead worker bees were removed each day. Three biological replicas were used in this experiment.

Measurement of spore load in workers' midguts

Using the method described by our team (Geng 2021), the spore load in the workers' midguts during the *N. ceranae* infection process was counted continuously. In brief, (1) after inoculation with *N. ceranae*, a single worker in the treatment group was taken from the cage every day, and their midgut was then dissected using a clean ophthalmic forceps and transferred into a sterile 1.5 μL Eppendorf (EP) tube. (2) Next, 200 μL of sterile water was added to the EP tube, and then full grinding (Meibi, Zhejiang, China) with an automatic high throughput tissue grinder was carried out. (3) Sterile water (800 μL) was added to the grinding liquid and then fully mixed by repeated shaking. (4) Using a micropipette, 100 μL of the aforementioned solution was carefully observed using a hemocytometer plate (Qiujing Shanghai, China) under an optical microscope (CSOIF, Shanghai, China), and then spore counting was conducted.

Detection of host sucrose solution consumption

Daily consumption of sucrose solution by each worker in the *N. ceranae*-inoculated and un-inoculated groups was analyzed using a previously described method (Liu et al. 2020; Di et al. 2016). The feeder containing 4 mL of sucrose solution was weighed, and the weight was recorded as A. To avoid error due to repeated changing of feeders, the feeder was weighed every 24 h thereafter, and the weight was recorded as B. The sucrose solution in the feeder was replaced daily. Sucrose solution consumption was calculated per worker per day following the formula (B-A)/number of survivors in the *N. ceranae*-inoculated group (or un-inoculated group).

Paraffin section, hematoxylin eosin (HE) staining, and microscopic observation of workers' midgut tissues

At 7 days post inoculation (dpi), 8 dpi, 9 dpi, and 10 dpi, midgut tissues of workers in the *N. ceranae*-inoculated group and un-inoculated group were dissected, respectively. Next, 4% paraformaldehyde was used to fix the midgut tissues. By using an embedding center (Junjie, Wuhan, China) and a microtome (Leica, Nussloch, Germany), paraffin sections of midguts were stained with HE stained by Shanghai Sangon Biological Engineering Co. Ltd., and then detected under an optical microscope with digital camera (SOPTOP, Shanghai, China).

At 7, 8, 9 and 10 days post inoculation (dpi), midgut tissues form workers in the *N. ceranae*-inoculated and un-inoculated groups were dissected. Next, 4% paraformaldehyde was used to fix the midgut tissues. By using an embedding center (Junjie, Wuhan, China) and a microtome (Leica, Nussloch, Germany), paraffin sections of midguts were stained with HE by Shanghai Sangon Biological Engineering Co. Ltd., and then detected under an optical microscope with a digital camera (SOPTOP, Shanghai, China).

Survival rate of workers

Following the protocol mentioned above, *A. c. cerana* workers were inoculated with *N. ceranae* spores and artificially reared. In another group, workers were fed with sucrose solution free of microsporidian spores. After inoculation, the numbers of dead workers from the *N. ceranae*-inoculated and un-inoculated groups were recorded daily until 20 days post inoculation. This was followed by calculated of the survival rate and data analysis using GraphPad Prism 6.0 software (GraphPad, San Diego, CA, USA).

Statistics

Statistical analysis of the data was performed using SPSS software version. 21 (IBM, Armonk, NY, USA) and GraphPad Prism 6.0 software. Data were presented using mean ± standard deviation (SD), and the significance of the microsporidian spore load and host sucrose solution consumption was analyzed using One-Way ANOVA. *P* values were corrected with the Bonferroni test. The log-rank (Mantel-Cox) test was used to analyze the host survival rate. $P < 0.05$ was considered to indicate a statistically significant difference, and $P < 0.01$ was considered to indicate a highly significant difference.

Results

Dynamics of *N. ceranae* spore load in the midguts of *A. c. ceranae* workers

The spore counting results suggest that, in comparison with the spore load at 0 dpi, the spore load in the host midgut decreased from 1 dpi ($df = 5$, $P = 0.0002$) to 2 dpi ($df = 5$, $P < 0.0001$), whereas an elevated trend was displayed from 2 dpi ($df = 5$, $P < 0.0001$) to 13 dpi ($df = 5$, $P = 0.0376$); however, the spore load decreased from 13 dpi ($df = 5$, $P = 0.0376$) to 14 dpi ($df = 5$, $P = 0.0194$) (Fig. 2).

A non-significant difference in sucrose solution consumption between *N. ceranae*-inoculated and un-inoculated workers was found

The sucrose solution consumption of *A. c. cerana* workers was calculated, and it was found that from 1 dpi ($P = 0.6702$) to 20 dpi ($P = 0.5332$), the consumption of workers in the *N. ceranae*-inoculated group was nearly always higher than that of workers in the un-inoculated group, except for at 3 dpi ($P = 0.4859$), 17 dpi ($P = 0.7745$), and 20 dpi ($P = 0.5332$) (Fig. 3). The average sucrose consumption of *N. ceranae*-inoculated and un-inoculated workers was 0.0357 ± 0.0136 and 0.0323 ± 0.0066 g per day, respectively.

However, a significant difference in sucrose solution consumption between these two groups was only observed at 5 dpi ($P = 0.0422$) and 13 dpi ($P = 0.0286$) (Fig. 3).

The midgut epithelial cell structure of A. c. cerana **workers was damaged by** N. ceranae **infection**

Microscopic observation of paraffin sections showed darkly stained microsporidia in the midgut epithelial cells of *N. ceranae*-inoculated workers from 7 dpi to 10 dpi, whereas no microsporidia were detected in the cells of un-inoculated workers (Fig. 4). Additionally, the number of microsporidian spores in the host cells gradually increased with extension of the infection time (Fig. 4). Moreover, the boundaries of un-inoculated host epithelial cells were found to be intact and the darkly stained nuclei were clear, while the boundaries of midgut epithelial cells of *N. ceranae*-inoculated workers were blurred, the nuclei had almost disappeared, and the nucleic acid substances were diffused (Fig. 4).

The lifespan of *A. c. cerana* workers was shortened by *N. ceranae* infection

The survival rate statistics indicate that the survival rates of workers in both the *N. ceranae*-inoculated and un-inoculated groups at 1 to 5 dpi were pretty high, and there was little difference between groups (Fig. 5). The survival rates of both groups started to decrease at 5 dpi (Fig. 5). From 5 to 11 dpi, the survival rate of workers in the *N. ceranae*-inoculated group was lower than that in the un-inoculated group, whereas the survival of workers in the *N. ceranae*-inoculated group was significantly lower than that in the un-inoculated groups from 11 to 20 dpi (Fig. 5).

Discussion

Currently, our understanding of the interaction between the Asian honeybee and *N. ceranae* is very limited. Previous work suggested that both *A. mellifera* and *A. cerana* workers could be effectively infected by inoculation with *N. ceranae* spores (Tsai et al. 2003; Jack et al. 2016). It has been suggested that in *A. mellifera* workers' midguts, the spore load of *N. ceranae* continuously increases from 7 to 14 dpi and from 12 dpi to 20 dpi (Huang and Solter 2013; Charbonneau et al. 2016). Here, we detected that the spore load of *N. ceranae* increased from 2 to 13 dpi (Fig. 2), indicating continuous proliferation of *N. ceranae* in host cells. In detail, the spore load of *N. ceranae* in the workers' midguts was found to decrease from 1 to 2 dpi, and then increase from 2 to 5 dpi; thereafter, it decreased from 5 to 6 dpi and continuously elevated from 6 to 9 dpi (Fig. 2). It is speculated that the life cycle of *N. ceranae* in the midgut epithelial cells of *A. c. cerana* workers is approximately five days, longer than that of *A. mellifera* workers infected by *N. ceranae* (about four days). To a certain extent, this shows that *N. ceranae* is more virulent against *A. mellifera* workers than *A. cerana* workers, which is in accordance with the phenomenon that *A. mellifera* workers are usually heavily infected by *N. ceranae* (Higes et al. 2007). Additionally, microscopic observation of paraffin sections showed that the number of microsporidia in the midgut epithelial cells increased from 7 to 10 dpi (Fig. 4), further indicating continuous multiplication of *N. ceranae* during the infection process. These results offer a solid basis for further investigations of *N. ceranae* infection of *A.*

cerana workers, for example, functional studies on fungal virulence factor-associated genes and development of the RNAi-based control strategy.

N. ceranae infection causes damage to the structure of midgut epithelial cells in *A. mellifera* workers (García-Palencia et al. 2010). Darkly stained microsporidia were clearly detected in the midgut epithelial cells of *N. ceranae*-inoculated workers from 7 to 10 dpi (Fig. 4). The boundaries of midgut epithelial cells in *N. ceranae*-inoculated workers were blurred, and the nuclei had almost disappeared, with the nucleic acid substances dispersed around the cytoplasm (Fig. 4). This suggests that *N. ceranae* infection damages the structure of the midgut epithelial cells in *A. c. cerana* workers.

Paris *et al* (Paris et al. 2017). assessed the survival rate and eating behavior of *A. mellifera* workers infected by *N. ceranae* for a period of 1 to 22 days. The results showed that the survival rate of infected workers was significantly lower than that of un-infected workers. In a previous study, we observed that the mortality rate of *A. mellifera* workers inoculated with *N. ceranae* spores gradually increased over time, and the mortality rate of *N. ceranae*-inoculated workers was significantly higher than that of un-inoculated workers at 7 and 10 dpi (Chen et al. 2019). It has been documented that *N. ceranae* infection significantly increases the mortality rate of *A. cerana* workers (Huang et al. 2018). In this study, the survival rates of workers in both the *N. ceranae*-inoculated and un-inoculated groups were pretty high from 1 to 5 dpi (Fig. 5), implying that *N. ceranae* had little influence on host longevity at the early stage of infection. In addition, from 5 to 20 dpi, the survival rate of *N. ceranae*-inoculated workers was always lower than that of un-inoculated workers, and the difference was significant at 11 to 20 dpi (Fig. 5), indicating that *N. ceranae* infection shortened the lifespan of *A. cerana* workers, similar to the finding of Huang *et al* (Huang et al. 2018). In conclusion, *N. ceranae* is able to exert negative influences on the midgut epithelial cell structure and the lifespan of *A. c. cerana* workers during the infection process.

Conclusions

Taken together. We conducted an in-depth investigation of *N. ceranae* infection of *Apis cerana cerana* workers and the resulting impacts on host sucrose solution consumption, midgut epithelial cell structure, and lifespan. Our results indicate that the spore load continuously elevates as *N. ceranae* proliferates, causing damage to the host midgut epithelial cell structure and decreasing the host's lifespan. The findings of this current work provide a solid foundation for dissecting the mechanism underlying *N. ceranae* infection and the interaction between *N. ceranae* and *A. cerana*.

Declarations

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Conflicts of Interest The authors declare that they have no conflict of interest.

Availability of data and material Not applicable

Author Contributions R.G. and D.C. designed this research; Q.L., M.S. and X.F. contributed to the writing of the article; Q.L., M.S., X.F., W.Z., D.Z., Y.H., Z.W., K.Z., K.Y., H.Z., Y.S. and Z.F., conducted experiments and data analyses. R.G. and D.C. supervised the study and preparation of the manuscript.

Ethics approval Not applicable

Consent to participate Not applicable

Consent for publication Not applicable

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Figures

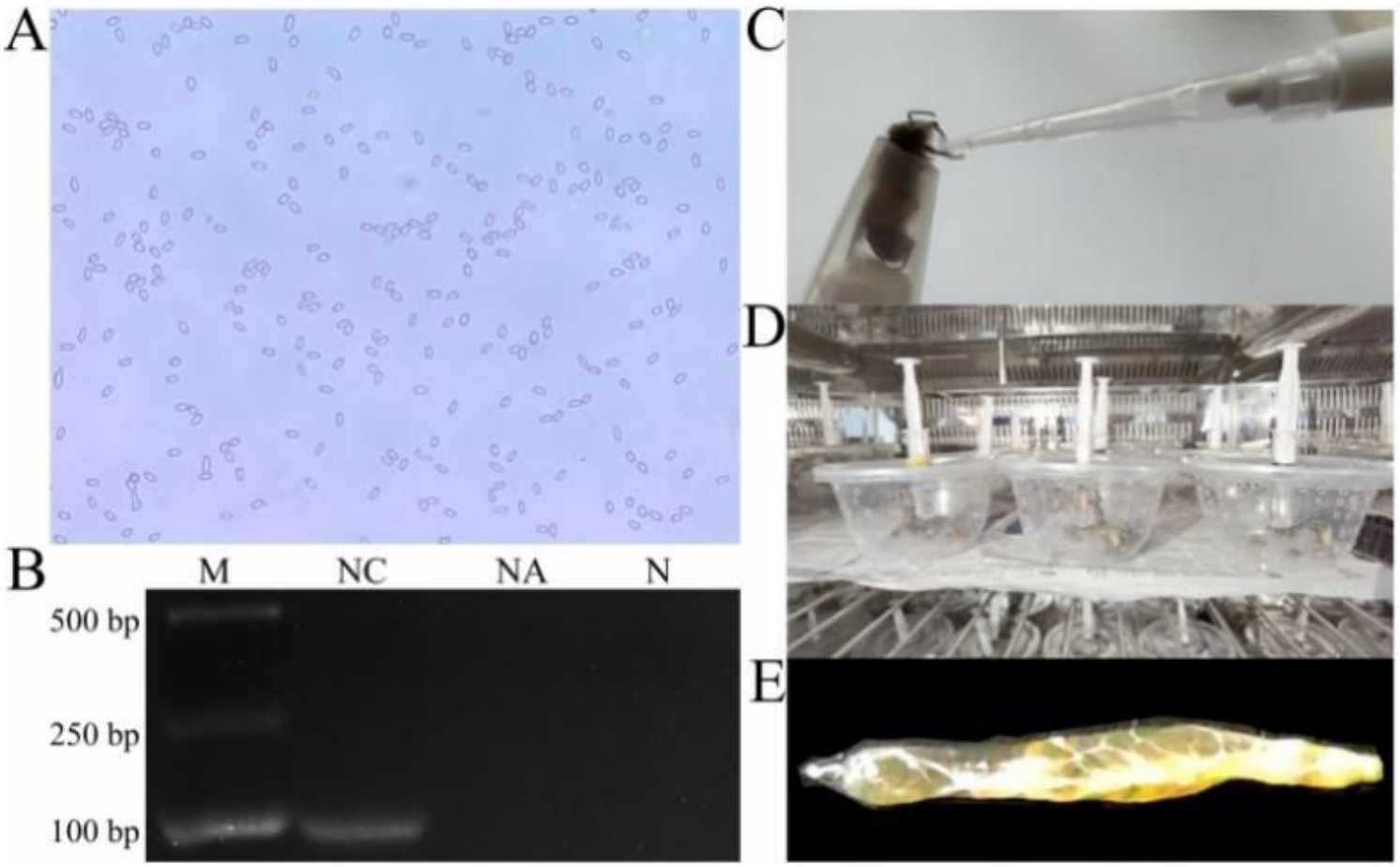

Figure 1

(A) Microscopic observation of *N. ceranae* spores derived from Percoll discontinuous density centrifugation (400 times amplification). (B) Agarose gel electrophoresis for PCR amplification products from purified spores. Lane M: DNA marker; Lane NC: specific primers for *N. ceranae*; Lane NA: specific primers for *N. apis*; Lane N: sterile water (negative control). (C) Artificial inoculation of a fixed worker using a pipette. (D) Artificial rearing of workers kept in plastic cages in incubator. (E) A midgut tissue of a worker after dissection.

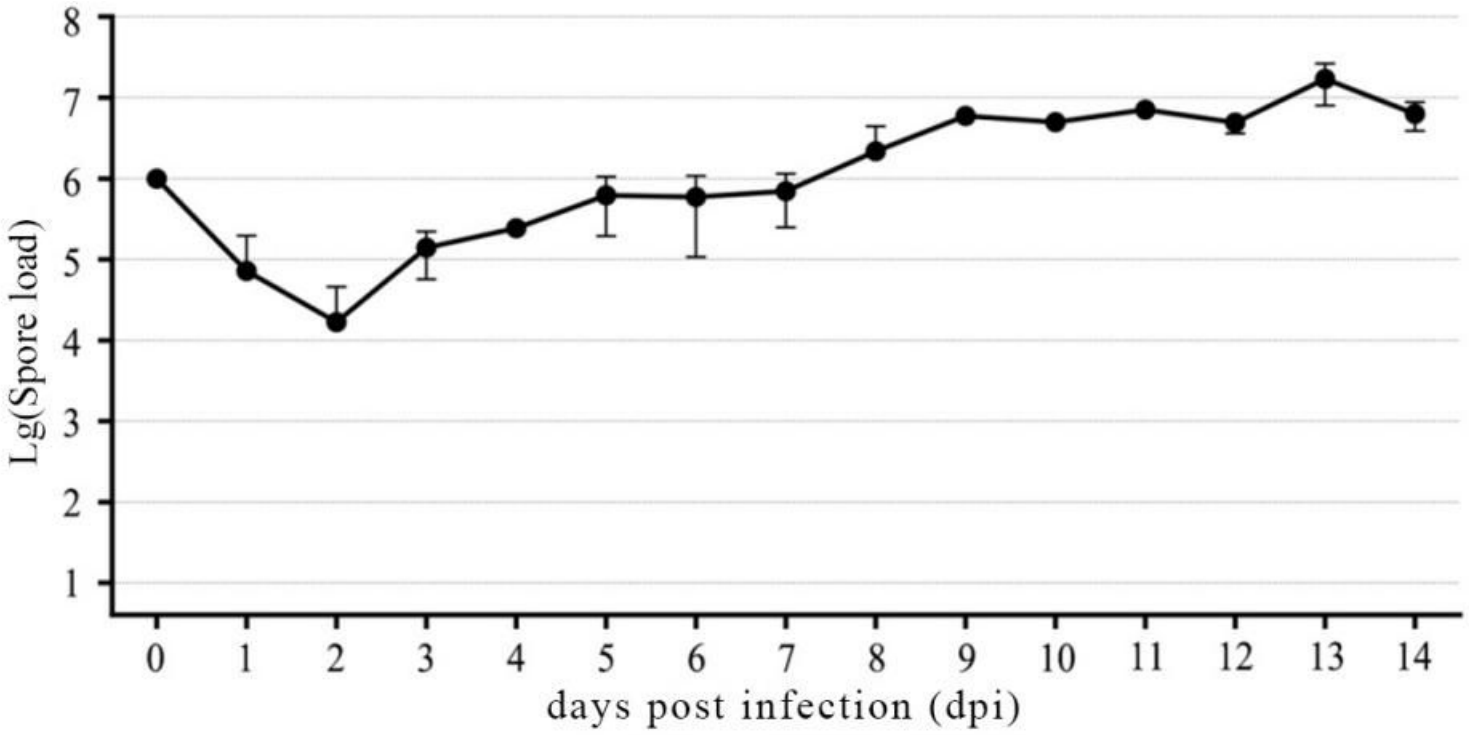

Figure 2

Spore load of *N. ceranae* in the *A. c. cerana* workers' midguts after inoculation. The whiskers indicate the mean ± standard deviation (SD).

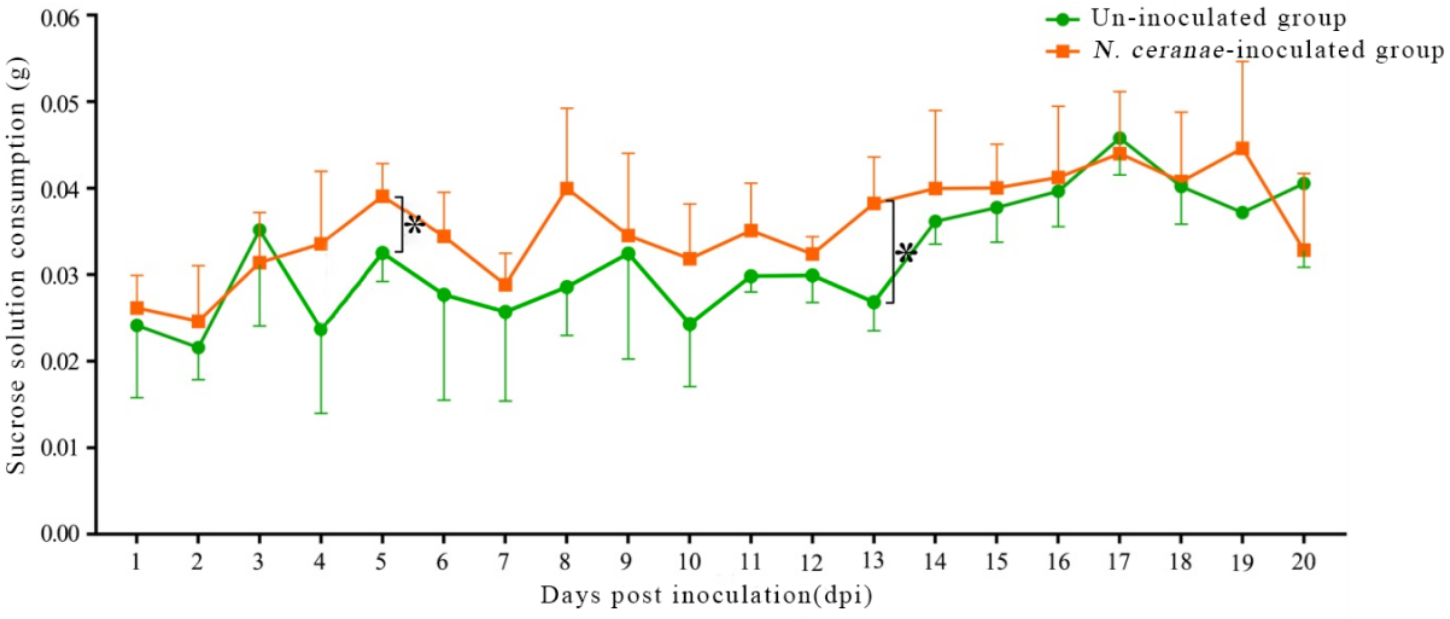

Figure 3

Sucrose solution consumption of *N. ceranae*-inoculated and un-inoculated workers. The whiskers indicate the mean ± standard deviation (SD). Data were analyzed using the ANOVA test; * indicates $P<0.05$. *P* value was corrected using Bonferroni test.

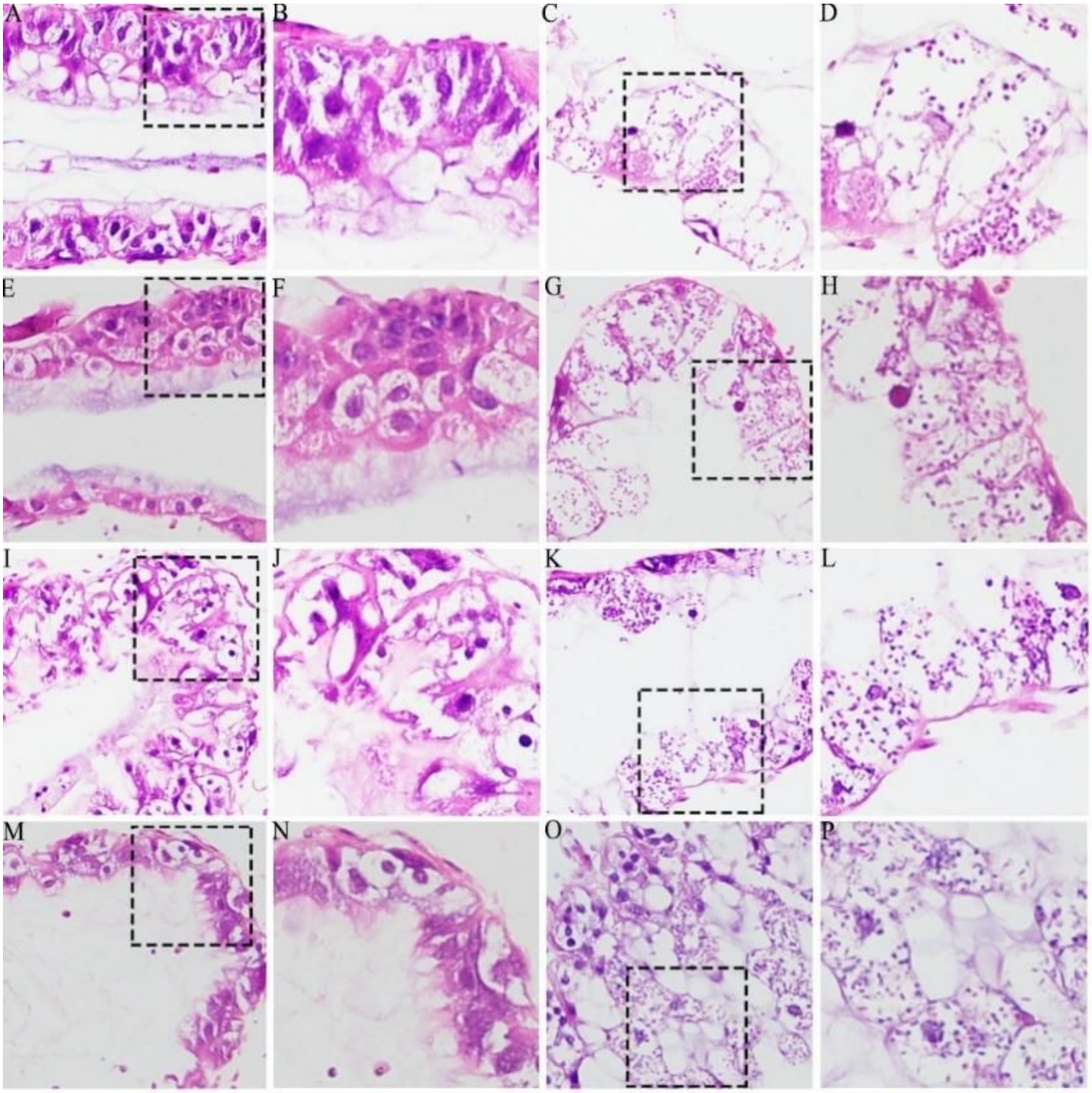

Figure 4

Microscopic observation of paraffin sections of un-infected and *N. ceranae*-infected *A. c. cerana* workers' midguts. A-B: Midgut tissue of un-inoculated worker at 7 dpi without spores; C-D: Midgut tissue of inoculated worker at 7 dpi with *N. ceranae* spores; E-F: Midgut tissue of un-inoculated worker at 8 dpi without spores; G-H: Midgut tissue of inoculated worker at 8 dpi with *N. ceranae* spores; I-J: Midgut tissue of un-inoculated worker at 9 dpi without spores; K-L: Midgut tissue of inoculated worker at 9 dpi with *N. ceranae* spores; M-N: Midgut tissue of un-inoculated worker at 10 dpi without spores; O-P: Midgut tissue

of inoculated worker at 10 dpi with *N. ceranae* spores. A, C, E, G, I, K, M, and O were microscopic fields under 200 times amplification, while B, D, F, H, J, N, and P were microscopic fields under 400 times amplification. Black dashed boxes show the selected region for observation under 200 times amplification.

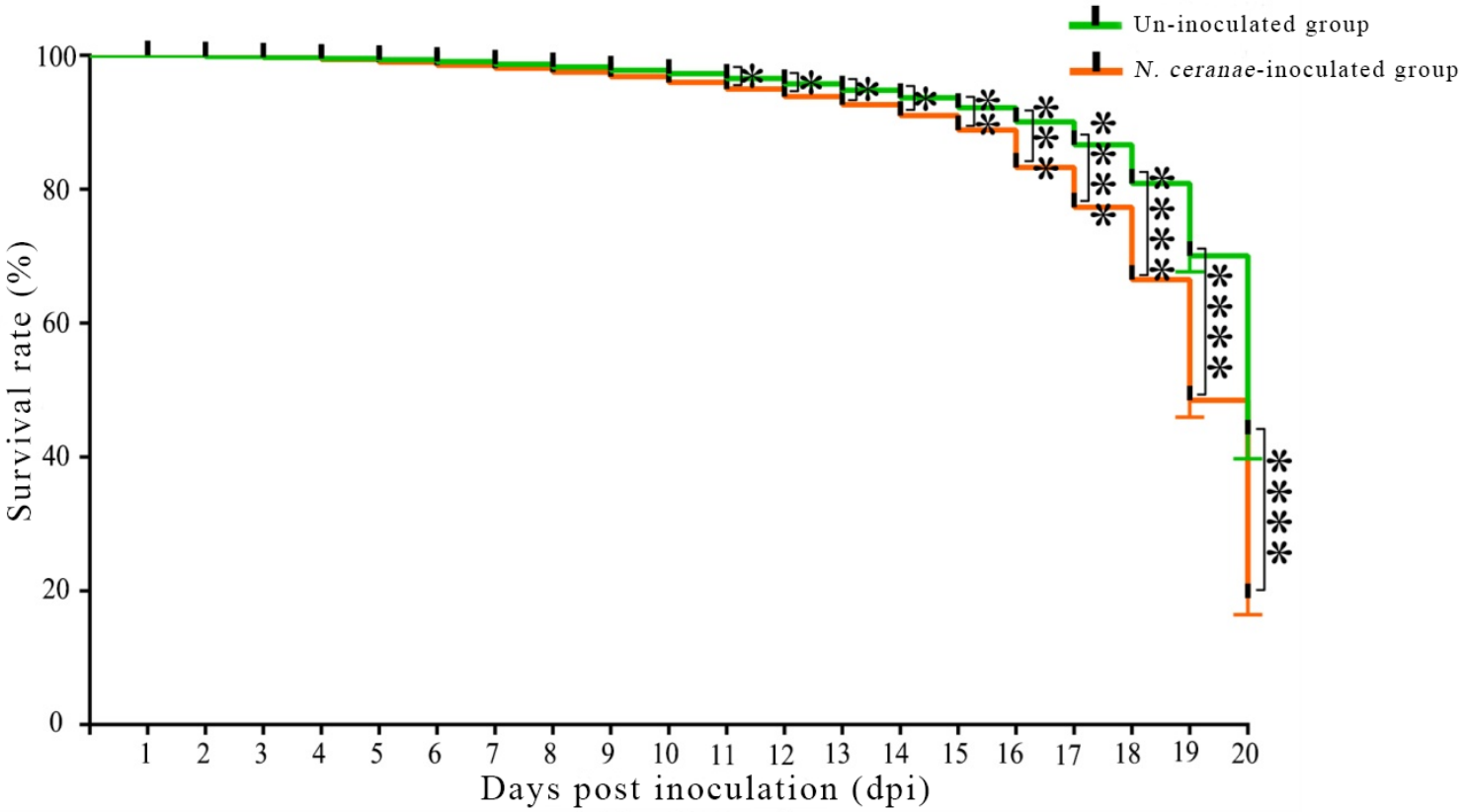

Figure 5

Survival rate of *A. c. cerana* workers after un-inoculation and inoculation with *N. ceranae* spores. Data were analyzed using Log-rank (Mantel-Cox) test; * indicates $P<0.05$, ** indicates $P<0.01$, *** indicates $P<0.001$, **** indicates $P<0.0001$.