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Short Communication

Infection dynamics of *Nosema ceranae* in honey bee midgut and host cell apoptosis

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A B S T R A C T

Nosema ceranae is an intracellular microsporidian parasite that infects epithelial cells of the honey bee (*Apis mellifera*) midgut. Previous studies have shown that *Nosema* may alter cell renewal and apoptosis in honey bees. We found that the amount of apoptotic cells progressively declines from the anterior towards posterior regions of the midgut in *Nosema*-infected sensitive bees. There was no such pattern in the infected *Nosema* tolerant honey bees and controls. These data provide additional evidence that *N. ceranae* appears to alter apoptosis in its host cells for its own advantage.

1. Introduction

The microsporidian parasite *Nosema ceranae* is a globally emerging pathogen that infects honey bees (*Apis mellifera*) (Klee et al., 2007). Hence, the effects of *N. ceranae* on its host and host immune defenses have been studied in considerable detail over the last decade (Fries et al., 2013; Higes et al., 2013b; Kurze et al., 2016c). *Nosema* is primarily transmitted via the fecal-oral route between adult bees and establishes an infection in the midgut epithelium (Fries, 2010). Over the course of infection, *N. ceranae* has been shown to induce multiple physiological alterations in its host, including processes involved in the regulation of programmed cell death (PCD) (Doublet et al., 2017; Higes et al., 2013a; Kurze et al., 2015; Martin-Hernandez et al., 2017) and energy metabolism (Kurze et al., 2016b; Mayack and Naug, 2009; Vidau et al., 2014). This might be not surprising as microsporidia rely on their host metabolism for supply of energy resources (Keeling, 2009). A breeding program selecting against *Nosema* over 20 years resulted in a *Nosema* tolerant honey bee lineage (Hatjina et al., 2014). Although *Nosema* prevalence reduced from 60–80% down to about 10% in selected colonies (Hatjina et al., 2014), individual bees were still susceptible to both *Nosema apis* and *N. ceranae* in laboratory

experiments (Kurze et al., 2016b). Interestingly, Huang et al. (2012) showed a higher survival and increased immune responses in those tolerant bees compared to sensitive bees from an unselected lineage, despite suffering similar infection intensities of *N. ceranae*. In subsequent experiments, infected worker bees from this *Nosema* tolerant lineage appeared to neither experience decreased rates of apoptosis (one form of PCD) (Kurze et al., 2015) nor energetic stress (Kurze et al., 2016b). A similar pattern was also found when comparing the midgut proteomes between infected workers of both the *Nosema* tolerant and a *Nosema* sensitive lineage (Kurze et al., 2016a).

Although the midgut is clearly the major site of the interaction between *Nosema* and honey bee, specific data on the actual dynamics along the host's midgut epithelium are lacking. In this study, we focused on patterns of apoptosis in response to *N. ceranae* infection in different regions of the midgut after six days post infection (dpi). We quantified the rates of apoptosis in longitudinal sections of the midgut in *Nosema* tolerant and sensitive bees to gain a better understanding of the local effects caused by *N. ceranae*.

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2. Materials and methods

2.1. Experimental inoculation

We conducted three experimental replicates at the Unité de Recherche Abeilles et Environnement de l'INRA Avignon (France) as described previously (Kurze et al., 2015). Briefly, twenty newly emerged workers (< 24 h old) from both *Nosema* sensitive (SN) and *Nosema* tolerant colonies (TN, originally from an apiary in Aarhus, Denmark) were individually fed with freshly extracted and purified 50,000 *N. ceranae* spores μl^{-1} in 2 μl of 50% (w/v) sucrose solution. Uninfected controls of the sensitive (SC) and tolerant (TC) strains were fed with 2 μl sucrose solution without spores. We did not include bees in the experiments that had not consumed the entire inoculum. Each treatment group (i.e. SC, TC, SN and TN) was separately housed in autoclaved cages (10 × 10 × 5.5 cm) with a piece of clean wax foundation in an incubator at 34 ± 1 °C, 60% relative humidity and provided with 50% (w/v) sucrose solution *ad libitum*. Experiments were terminated after six days' post-infection (dpi).

2.2. Spore counts

We dissected midguts (ventriculi without rectum) for a random subset of 5 bees for each treatment group and cage replicate. Subsequently, midguts were homogenized in 1000 μl of distilled water and the numbers of mature spores were counted with a Fuchs-Rosenthal hemocytometer using a phase-contrast microscope at × 400 magnification.

2.3. Immunohistochemistry

We randomly chose three bees per treatment group and replicate to dissect their midguts (ventriculi without rectum). These were subsequently fixed in 4% buffered formaldehyde for 24 h at 8 °C and embedded in paraffin according to standard methods. To visualize apoptotic cell at the early stage, we conducted TUNEL (Terminal deoxynucleotide transferase mediated X-dUTP nick endlabelling) assays (In Situ Cell Death Detection Kit, Roche Diagnostics GmbH, Mannheim, Germany) on 7 μm thick longitudinal sections following manufacturer's recommendations. Prior to the TUNEL reaction, endogenous peroxidase activity was blocked (Dual Endogenous Enzyme Block, Dako Denmark, Glostrup, Denmark), followed by permeabilisation step using nucleases-free 10 μg μl^{-1} proteinase K in 10 mM Tris/HCl pH 7.5 for 20 min at room temperature. Then we rinsed samples twice in PBS (phosphate-buffered saline). After an incubation time of 1 h at 37 °C in the dark, the TUNEL reaction was stopped by rinsing the samples three times in PBS and follow by counterstaining reaction with 1 μg ml^{-1} DAPI (4',6-diamidino-2-phenylindole) (Sigma-Aldrich, St. Louis, United States). We quantified the relative number of apoptotic cells (TUNEL+ve, Fig. 1) in the anterior, central and posterior regions of the midgut epithelium as described previously (Kurze et al., 2015). Briefly, we compiled four images per region visualizing the TUNEL and DAPI stain (microscope Axiovert25, camera AxioCam, software Axiovision 4.6; all from Zeiss, Jena, Germany) and images were automatically analyzed in Fiji (Schindelin et al., 2012) (Fig. 1). Only samples with undisrupted epithelium from anterior until posterior midgut (i.e. due to microtome sectioning) and good image quality have been included into the analysis. Final samples sizes were n = 9 (SC), n = 7 (SN), n = 7 (TC) and n = 7 (TN).

2.4. Statistics

All statistical analyses and data plotting (mean ± 95% confidence intervals, CI) were carried out in R (v.3.0.2). Spore load between infected groups was tested using Welch's two-tailed t-test. To test the effects on the apoptosis ratio, we ran Linear Mixed-Effects Models

(LMMs) using the *lmer* function of the *lme4* package (v.1.1-12). If a model was found to be unstable with all interactions included, we removed non-significant interactions step-wise. Model validity was tested by comparing full models to their null models without any fixed factors included using maximum likelihood ratio test. Additionally, we checked the data for normality and residuals. The final model including the treatment, bee lineage, midgut regions and their interactions as fixed factors and nested them under each individual bee as random factor. We used the *glht* function of the *multcomp* package (v.1.3-2.) for between group comparisons, correcting for multiple testing using the 'false discover rate'.

3. Results and discussion

The insect midgut is a dynamic organ, which is essential for immune defense, digestion and detoxification. To maintain these functions, senescent, damaged and infected cells are replaced through PCD (including apoptosis) and asymmetric cell division (Lee et al., 2017). In our experiment, we found that one-week old uninfected control bees (SC and TC) maintained overall an average cell turnover of 40% ± 3 CI (n = 16) from the anterior to the posterior part of the midgut (Fig. 2). Despite a slight reduction of apoptosis in the posterior midgut (LMM: estimate ± s.e. = -0.072 ± 0.032, p < 0.05; Fig. 2), we found the strongest effect for the interactions among bee lineages, midgut regions and treatments (LMM: 80.197 ± 0.070, p < 0.01). There was a highly significant difference in apoptosis between the anterior with 40% ± 12 CI and posterior region with 22% ± 10 CI (n = 7) in SN (0.197 ± 0.036, p < 0.0001; Fig. 2). The rate of apoptosis was also slightly lower when comparing the anterior with central regions in those bees (0.097 ± 0.036, p < 0.05; Fig. 2). Furthermore, apoptosis in the central and posterior midgut were significantly lower in SN compared to their controls SC (-0.143 ± 0.046, p < 0.05 and -0.138 ± 0.046, p < 0.05 respectively; Fig. 2). As infestation appeared to increase from the anterior to the posterior epithelium at this relatively early stage of infection (Fries, 1988), our results strongly support previous suggestions that *N. ceranae* inhibits host cell apoptosis (Higes et al., 2013a; Kurze et al., 2015; Martin-Hernandez et al., 2017). An important role of PCD in bee-*Nosema* interactions was also indicated by proteomic and transcriptomic studies (Doublet et al., 2017; Dussaubat et al., 2012; Holt et al., 2013; Kurze et al., 2016a).

Although overall there was no difference between the numbers of *Nosema* spores in sensitive (SN: 6.0 ± 1.2 × 10⁶ spores; n = 14) and tolerant honeybees (TN: 8.0 ± 1.4 × 10⁶ spores; n = 15) after 6 dpi (t-test: t = 1.115, d.f. = 26.77, p > 0.05), we did not observe reduced apoptosis along the epithelium in TN (36% ± 3 CI; 0.019 ± 0.036, p > 0.05; Fig. 2). Despite severe infection levels, TN maintained similarly high levels of apoptosis in the posterior region (38% ± 2 CI, n = 7) as control bees (0.050 ± 0.049, p > 0.05), but their levels were significantly higher as in SN (0.156 ± 0.049, p < 0.05; Fig. 2). The high apoptosis levels of midgut epithelium in the *Nosema* tolerant bee lineage might have been suggested to reduce the infection intensity over a longer period through defecation outside the hive and thereby also reducing the risk of horizontal transmission (Kurze et al., 2015).

Since the posterior insect midgut is an important site for virus acquisition (for example in mosquitos and aphids, McGee et al., 2010; Reinbold et al., 2003; Vaidyanathan and Scott, 2006), reduced apoptosis of almost 50% in SN may facilitate secondary infections that could lead to increased mortality. Although *N. ceranae* has been suggested to promote virus infections by suppressing the host immune system (Antúnez et al., 2009), it has been recently shown to have also negative effects on viruses (Doublet et al., 2015). The study of any interactions between *Nosema* with other agents in the gut microbiome is therefore clearly a rewarding topic for future research. Nevertheless, the region-dependent dynamics of the interactions between *N. ceranae* and the host cell epithelium, are an important step towards understanding the pathophysiology of *Nosema* infections.

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Fig. 1. Detection and quantification of apoptotic cell in the midgut epithelium of honey bees infected with *N. ceranae*. (A) Typical DAPI and (B) TUNEL stained image. TUNEL assay is used to detect apoptotic nuclei and DAPI stains all nuclei in the epithelium. Arrows indicate TUNEL artefacts (false positive signals) that excluded from automated analysis using ImageJ. (C) Merged images and contrast analysis. (D) Binarization of TUNEL + ve (red) and TUNEL - ve (white) nuclei was followed by automatic nuclei counting. The frequency of apoptotic cells as calculated as the numbers of TUNEL + ve relation to all nuclei (DAPI + ve). Scale bars = 50 µm.

Fig. 2. Differences of apoptosis in the midgut of *Nosema* sensitive and tolerant honey bees after 6 dpi. Mean ratios of apoptosis $\pm$ 95% confidence intervals (CI) for anterior (white), central (light grey) and posterior (dark grey) midgut regions in both control and infected *Nosema* sensitive and tolerant bees. Significant differences are indicated as * for $p < 0.05$ and *** $p < 0.001$.

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Author contributions

The study was conceived and designed by CK, YLC, PK and RFAM. Experiments were conducted by CK; molecular work and analyses were carried out by CK, OL, TM and RFAM. Manuscript was written by CK, edited by RFAM, and reviewed by YLC, PK, TM, OL.

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