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Impact of the microsporidian *Nosema ceranae* on the gut epithelium renewal of the honeybee, *Apis mellifera*

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Abstract

The invasive microsporidian species, *Nosema ceranae*, causes nosemosis in honeybees and is suspected to be involved in Western honeybee (*Apis mellifera*) declines worldwide. The midgut of honeybees is the site of infection; the microsporidium can disturb the functioning of this organ and, thus, the bee physiology. Host defense against pathogens is not limited to resistance (i.e. the immune response) but also involves resilience. This process implies that the host can tolerate and repair damage inflicted by the infection– by the pathogen itself or by an excessive host immune response. Enterocyte damage caused by *N. ceranae* can be compensated by proliferation of intestinal stem cells (ISCs) that are under the control of multiple pathways. In the present study, we investigated the impact of *N. ceranae* on honeybee epithelium renewal by following the mitotic index of midgut stem cells during a 22-day *N. ceranae* infection. Fluorescence in situ hybridization (FISH) and immunostaining experiments were performed to follow the parasite proliferation/progression in the intestinal tissue, especially in the ISCs as they are key cells for the midgut homeostasis. We also monitored the transcriptomic profile of 7 genes coding for key proteins involved in pathways implicated in the gut epithelium renewal and homeostasis. We have shown for the first time that *N. ceranae* can negatively alter the gut epithelium renewal rate and disrupt some signaling pathways involved in the gut homeostasis. This alteration is correlated to a reduced longevity of *N. ceranae*-infected honeybees and we can assume that honeybee susceptibility to *N. ceranae* could be due to an impaired ability to repair gut damage.

I. Introduction

The western honeybee *Apis mellifera* is a beneficial insect of agronomic and environmental importance (Gallai *et al.*, 2009). For the past few decades, bee colony losses have been reported worldwide at alarming rates. The causes are likely to be multifactorial, involving predators, pesticides and pathogens (Vanengelsdorp and Meixner, 2010). Among them, the intestinal parasite *Nosema ceranae* is one of the most common pathogens in *A. mellifera* (Higes *et al.*, 2008). *N. ceranae* belongs to Microsporidia, a phylum of obligate intracellular eukaryotic parasites related to fungi with more than 1,500 described species infecting almost all animal phyla (Texier *et al.*, 2010; Stentiford *et al.*, 2016). Their hosts include numerous species of economic and ecological significance, including pollinators and farmed crustacean species (Stentiford *et al.*, 2016). Microsporidia are also considered to be emerging pathogens in humans (Texier *et al.*, 2010), and more recent studies have shown that their prevalence in immune competent humans is larger than expected (Sak *et al.*, 2011).

Nosemosis is a widespread disease that may be a serious threat for honeybee health. Two microsporidian species infect honeybees : *Nosema apis,* described in 1909 (Zander, 1909) and *N. ceranae* described in 1996, which originated from the Asian honeybee species *Apis cerana* (Fries *et al.*, 1996). *N. ceranae* evidently switched hosts from *A. cerana* to *A. mellifera* some decades ago, and spread in *A. mellifera* globally (Klee *et al.*, 2007). The pathogenicity and relative virulence of *N. ceranae* is still controversial; this parasite has been found both in little impacted honeybee colonies (Martín-Hernández *et al.*, 2011; Milbrath *et al.*, 2015) and in weakened colonies (Higes *et al.*, 2007). The major disorders described in infected honeybees are energetic stress (Mayack and Naug, 2009; Alaux *et al.*, 2010), behavioral fever (Campbell *et al.*, 2010), hormonal disorder (Dussaubat *et al.*, 2010; Dussaubat *et al.*, 2013; Holt *et al.*, 2013),

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immunosuppression (Antunez *et al.*, 2009; Alaux *et al.*, 2010) and a decrease in the insect lifespan (Higes *et al.*, 2008; Alaux *et al.*, 2010; Vidau *et al.*, 2011; Aufauvre *et al.*, 2012; Goblirsch *et al.*, 2013; Aufauvre *et al.*, 2014). *N. ceranae* can also sensitize the honeybees to chemical stressors (Alaux *et al.*, 2010; Vidau *et al.*, 2011; Aufauvre *et al.*, 2012; Pettis *et al.*, 2012; Wu *et al.*, 2012; Aufauvre *et al.*, 2014; Retschnig *et al.*, 2014) and can reduce the efficiency of acaricide treatment against *Varroa destructor* (Botías *et al.*, 2012), another major threat for honeybees.

The honeybee midgut is involved in immunity, digestion and detoxification. It is also the primary site of *N. ceranae* proliferation and could, thus, be impaired by this parasite. As described in the insect model *Drosophila melanogaster*, intestinal immune responses can be triggered to protect against colonization by pathogens; however, such defense can cost more energetically than repair of damage caused by the pathogens (Ferrandon, 2013). The host can alternatively use a resilience mechanism by increasing the gut epithelium renewal rate, which could compensate for damage caused by the parasite. This gut homeostasis is slightly regulated by different pathways involved in gut integrity and by the microbiota pressure (Buchon *et al.*, 2009). Ageing, oxidative stress and infections lead to enterocyte damage and cell death that are counterbalanced by the proliferation of intestinal stem cells (ISCs) (Jiang and Edgar, 2009; Biteau *et al.*, 2012; Ferrandon, 2013). The damage can be sensed by the Hippo and JNK pathways and signals are transmitted to ISCs, directly or by the unpaired cytokines (Upd1, 2 and 3) for gut renewal as demonstrated in *D. melanogaster* (Buchon *et al.*, 2009; Staley and Irvine, 2010; Osman *et al.*, 2012).

Previous transcriptomic and proteomic studies suggested that these gut renewal pathways may be impaired by *N. ceranae* infection (Dussaubat *et al.*, 2012; Vidau *et al.*, 2014). We thus

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hypothesized that the decrease in honeybee lifespan caused by *N. ceranae* infection could be due to an alteration of gut epithelium homeostasis through a perturbation of its renewal ability, thus impairing the capacity to repair the damages caused by the parasite.

In the present study, we investigated the impact of *N. ceranae* on the honeybee epithelium renewal by following the mitotic index of midgut stem cells during a 22-day *N. ceranae* infection. Fluorescence in situ hybridization (FISH) and immunostaining experiments were performed to follow the parasite proliferation/progression in the intestinal tissue, especially in the ISCs as they are key cells for the midgut homeostasis. We also monitored the transcriptomic profile of seven genes coding for key proteins involved in pathways implicated in the gut epithelium renewal and homeostasis. Overall, this study provides new data concerning the honeybee response to the gut parasite *N. ceranae* at both tissue and molecular levels.

II. __Material and methods__

1. Bees and Parasites

Experiments were performed using honeybees from one *A. mellifera* colony located in an apiary near Combronde, France. Two frames of sealed brood were collected from this colony and incubated in the dark at 33°C with 80% r.h. Emerging honeybees were collected and distributed into Pain-type cages, 130 bees/cage,. Honeybees were then individually inoculated by feeding with 3 µl of 50% (w/v) sugar syrup containing 50,000 freshly purified spores of *N. ceranae* (Vidau *et al*. 2011). Control honeybees were also individually fed with 3 µL of sugar syrup but without *N. ceranae* spores. Four cages of each group (e.g., *N. ceranae*-infected or uninfected honeybees) were maintained in separate incubators in the dark at 33°C with 80% r.h. A 5-mm piece of Beeboost® (Phero Tech, Inc., Canada) that released five queen mandibular pheromones

was placed in each cage to simulate the hive environment. During the experiment, honeybees were fed *ad libitum* with 50% (w/v) sugar syrup supplemented with 1% (w/v) Provita'Bee (Vetopharm Pro). On a daily basis, dead bees were removed, counted and the sugar syrup consumption was quantified. The spore concentration in the rectum of bees collected at 7, 14 or 21 days post-infection was determined using a haemocytometer chamber (Higes *et al.*, 2007).

2. RNA extraction

For each sampling time point, 10 honeybee midguts per cage were dissected on ice, pooled and immediately immersed in liquid nitrogen, providing four pools of RNA samples for each the infected and uninfected bees. Collected midguts were homogenized in 500 μL TRIzol Reagent (Life Technologies) using a microtube pestle. The aqueous phase was supplemented with 0.015 volume of 2-mercaptoethanol, one volume of 70% ethanol and transferred to a RNeasy spin column (RNeasy Mini Kit, Qiagen); total RNA was isolated according to the manufacturer's recommendations. Genomic DNA was removed twice during each RNA extraction using the RNA-free DNase kit (Qiagen). Both RNA integrity and quantity were evaluated using the 2200 TapeStation (Agilent) with D1000 screen tape. Only RNA extracts with a RIN score > 8 were used for further experiments. For each uninfected and infected bees, the three replicates with the best RIN scores were retained and used for further experiments.

3. Real-Time Quantitative PCR (RT-qPCR)

cDNA synthesis was performed according to Aufauvre *et al*. (2014) in a 20 μL reaction volume containing 1 μg of total RNA, 0.5 mM of each dNTP and 200 U of SuperScript III Reverse Transcriptase (Life Technologies). For each sample, a control with no reverse-transcriptase (No-RT) was done to control for DNA contaminants. All RT-qPCR analyses were performed in 96-well plates on a Mastercycler Realplex 2 thermocycler (Eppendorf) monitored by the Realplex

software (version 1.5) using the primers and conditions listed in Table S1. All primers (already published or newly designed) were previously validated according to their specificity, linearity, efficiency and amplification reproducibility (Table S1). qPCR reaction mixtures consisted in 5 µL cDNA at 15 ng.µL^{-1}, the appropriate concentration of each primer and 10 µL of 2X Absolute Blue qPCR SYBR Green Mix (Thermo Scientific) in a final volume of 20 µL. Negative controls (without cDNA and No-RT) were included in each reaction. The PCR program consisted of an initial step at 95°C for 15 min, and 40 cycles of denaturation at 95°C for 15 s, specific annealing temperatures for 30 s and elongation at 72°C for 30 s. Fluorescence was measured in each cycle after the elongation step. The specificity of the reaction was checked by analyzing the melting curve of the final amplified products. The amplification results were expressed as the threshold cycle number (CT), which represents the number of cycles needed to generate a fluorescent signal greater than a predefined threshold. CT values were normalized by subtracting the CT value of the ribosomal protein S5 (RpS5), a housekeeping gene (Evans and Wheeler, 2000; Evans, 2004), from the corresponding cDNA sample. For each gene, data were analyzed using the QIAGEN Relative Expression Software Tool 2009 (REST 2009) (Pfaffl *et al.*, 2002), a value P(H1) ≤ 0.05 being accepted as significant.

4. ISC Proliferation Assay

We adapted a protocol of Ward *et al*. (2008) with a few modifications to assay the proliferation rate of the intestinal cells using immune histochemical labelling of the thymidine analog 5-bromo-2-deoxyuridine (BrdU) incorporated into newly synthesized DNA. One day before each sampling point, three bees per cage were randomly selected leading to 12 samples of each the infected and uninfected bees. These bees were individually fed with 5 µl of 5 mg/ml BrdU (Sigma) in 50% (w/v) sugar syrup, and placed in new cages in the dark at 33°C with 80% r.h for

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12 h in order to allow BrdU incorporation. Living individuals were then selected for further analysis. Dissected midguts were rinsed with PBS, fixed in Carnoy's fixative for 1 h at RT, then dehydrated and embedded in paraffin for sectioning. Sections (6 µm) were dewaxed in Histoclear, rehydrated via a graded alcohol series and permeabilized with 0.01% Triton X100 in PBS (PBS-T) for 5 min. Endogenous peroxidases were inhibited with 0.3% H_2O_2 for 30 min and DNA was denatured with 2N hypochloric acid in PBS-T for 1 h. After 3 washes in PBS-T, sections were blocked with 3% BSA in PBS-T supplemented with avidin (Avidin/Biotin Blocking kit, Vector Laboratories) for 1 h and incubated overnight with 1/250 anti-BrdU monoclonal antibody (Sigma-Aldrich) in PBS-T supplemented with biotin (avidin/biotin blocking kit, Vector Laboratories) at 4°C. After 3 washes in PBS-T, sections were incubated with 1/500 biotinylated goat anti-mouse secondary antibody (Invitrogen) in PBS-T for 1 h and washed 2 times in PBS-T. Amplification was performed by incubating the sample with 1/500 HRP-conjugated streptavidin (Invitrogen) for 30 min. After 2 washes in PBS-T, signals were revealed with Novared kit accordingly to manufacturer instructions (Vector Laboratories). After dehydration, sections were cleared in Histoclear and mounted with Cytoseal 60. Observations were done on a LEICA inverted microscope. For each individual, one randomly selected section was observed and all labelled nuclei and active centers of proliferation (crypts containing labelled nuclei) were counted. An active crypt was defined as containing at least one labelled nucleus. The effects of both *N. ceranae* infection and honeybee age on gut renewal rate were assessed by a two-way ANOVA after an Arcsin normalization of the counted data.

5. Expression of a recombinant NcPTP-2 in *Escherichia coli*

DNA was released from *N. ceranae* spores using the DNA easy Kit (Macherey Nagel). PCR primers designed to amplify a 510-bp genomic DNA fragment representing the amino acid region

104–273 of the *N. ceranae* polar tube protein 2 (NcPTP2) were NcPTP2-F : CG<u>GGATCC</u>GTAGCCAAGTTGCCACCT containing a *Bam*HI restriction site and NcPTP2-R : CG<u>GAATTC</u>GGGGTTCTGCATCCTTGTC with an *Eco*RI restriction site. PCR amplifications were performed using a Biorad DNA thermal cycler according to standard conditions. After denaturing DNA at 94°C for 3 min, 35 cycles were run with 20 s of denaturation at 94°C, 30 s of annealing at 54°C and 1 min of extension at 72°C, followed by a 10 min final extension step at 72°C. PCR products were analyzed by electrophoresis on 1% agarose gel and purified with QIA-quick gel extraction kit (Qiagen). After digestion with *Bam*HI and *Eco*RI, PCR products were cloned into a modified prokaryotic expression vector pGEX-4T1 (Pharmacia), in-frame with glutathione-*S*-transferase (GST) at the N-terminus and an eight histidine tag at the C-terminus. The resulting recombinant plasmid pGEX-4T1-PTP2 was introduced into the *E. coli* BL21+ strain. After induction with 0.5 mM IPTG for 3 h, bacterial proteins were solubilized in 2.5% SDS, 100 mM DTT and analyzed by SDS–PAGE on a 10% polyacrylamide gel. Swiss mice immunization with the expressed protein was carried out as described below.

6. Antibody production

Polyclonal antibodies were produced in Swiss mice against NcPTP2 after purification of the recombinant protein expressed in *E. coli* on glutathione sepharose 4B resin (GE Healthcare). Mice were injected intraperitoneally with purified proteins homogenized with Freund's adjuvant for the first injection and Freund's incomplete adjuvant for the next injections (at days 14, 21, and 28). Sera were collected 1 week after the last injection and stored at -20°C.

7. FISH and immunostaining

Infected midgut cells were observed after FISH with a specific probe (see below) and/or immunostaining using mouse anti-PTP2 antibodies. Infected honeybees were maintained for 6,

10 or 14 days as described in section 1. Guts were fixed in formaldehyde 4% for 12 h, rinsed twice in PBS and conserved at -20°C in 70% ethanol until use. Midguts were then embedded and sectioned as described in section 4 for FISH or immunostaining, with anti-PTP2 antibodies.

FISH: Paraffin-embedded sections were dewaxed and rehydrated through a series of decreasing ethanol concentrations. PBS was then gradually replaced by hybridization buffer (20 mM Tris-HCl pH 7.8, 0.9 M NaCl, 1X Denhardts solution and 0.01% SDS), as described in Dubuffet *et al* (2013). Hybridization was performed for 3 h at 45°C in the hybridization buffer containing 0.5 µM of a Cy3-labelled oligonucleotide probe designed to match specifically with *N. ceranae* small subunit RNA (Nc01 : 5'-TACCCGTCACAGCCTTGT-3') at the 5' end. After hybridization, sections were washed through a series of decreasing concentrations of hybridization buffer and temperature, counterstained with DAPI, and finally washed in PBS. Sections were mounted with Prolong Diamond (Molecular Probes) and 5 samples were observed with a LEICA epifluorescence microscope. Eighteen crypts (where are located the ISCs) were analyzed in total. Using this protocol, proliferative parasitic stages including meronts, sporonts and sporoblasts were stained.

Immunostaining: Embedded sections were dewaxed (as described previously) and incubated in PBS BSA 2% and 0,2% Triton X-100 for 1 h. Whole midguts were gradually rehydrated via alcohol series and then incubated in PBS BSA 2%, 0,2% Triton X-100 for 4 h. The samples were incubated overnight at 4°C with the anti-PTP2 antibodies (1/250). After three washes in PBS-T, the sections or whole midguts were incubated for 1 h at RT with Alexa Fluor 488 goat anti-mouse IgG secondary antibody (Molecular Probes) at a dilution 1:1,000 in PBS-T. After three washes, the sections were counterstained with DAPI. Sections were mounted with Prolong Diamond (Molecular Probes) and observed with a LEICA epifluorescence microscope.

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III. Results and Discussion

To analyze the honeybee response to *N. ceranae* infection, two sets (uninfected *vs* infected bees) of independent biological quadruplicates were used. No difference in cumulative sucrose consumption between control and infected bees was observed (Mann-Whitney test, p-value=0.886) (Figure S1). The honeybees were infected as 7.66×10^7 spores were counted in each rectum after 14 days post infection (dpi) (Figure S2). Significant mortality appeared at 14 dpi (10%) and reached nearly 50% at the end of the experiment (22 dpi), while only 13% mortality was observed at the end of the experiment for uninfected honeybees (Figure 1). These results are in agreement with previous studies that showed that honeybees infected with *Nosema* spp. have a shorter lifespan in both field and experimental conditions (Higes *et al.*, 2008; Alaux *et al.*, 2010; Vidau *et al.*, 2011; Aufauvre *et al.*, 2012). The increased mortality of infected bees could be due to starvation imposed by *N. ceranae* development as well as direct damage to the infected enterocytes (Mayack and Naug, 2009). It is also conceivable that the degeneration of epithelial ventricular cells and subsequent reduction of longevity could be linked to oxidative stress triggered by *N. ceranae* infection (Vidau *et al.*, 2014; Paris *et al.*, 2017). Taken together these factors may cause a potential lethal outcome if the epithelium is not capable of replacing the damaged cells as previously shown for the *Drosophila* gut (Buchon *et al.*, 2010).

To assess the impact of the infection on the intestinal epithelium renewal, the mitotic index of the ISCs was followed during the whole course of the infection using BrdU labelling. For each gut, the total number of crypts and the number of crypts containing at least one labelled nucleus were counted on one randomly-selected section, and the ratio of "active crypts" to "total crypts" was calculated. Unfortunately, for the 21 dpi time-point, the midgut tissues were so damaged that identifying the crypts was not possible.

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The statistical significance of the effects of the infection and the honeybee age on the epithelium renewal rate was evaluated by a two-way ANOVA. Our data revealed a significant effect of both the age of honey bee (p-value = 0.006) and infection with *N. ceranae* (p-value =0.0018) (Figure 2). Multiple comparison tests did not show significant difference between control and infected bees when comparing within an individual time point (data not shown), probably due to the high variability observed between each individual bee from the same group. However, these results indicate that the infection significantly downregulates ISC proliferation. This decrease was observed at all-time points of the kinetics except 3 dpi. It is not clear why no effect was found at this time point for any of the replicates, but a possible explanation may be that the observed impact on the renewal rate would be due to the presence of free spores in the intestinal lumen rather than to the infection of the cells themselves. At one day after inoculation, the spores used to infect the honeybees are absent in the intestinal lumen, but any potential reaction triggered by their passage, may continue to influence the epithelium until that point. In contrast, at 3 dpi, the infected cells did not yet start to release newly produced spores, as massive spore loads are usually not found before 6-7 dpi (Martin-Hernandez *et al.*, 2007) and any potential effect of the initial spore passage may have disappeared. This point raises the questions about the number of parasites needed to induce such an effect on intestinal tissue and/or whether the host response (such as the oxidative stress) could be responsible for this effect. Lower host cell proliferation due to microsporidian infection has previously been observed in the case of infection of E6 VERO cells by species of the *Encephalitozoon* genus (Scanlon *et al.*, 2004). Further studies are required to determine whether the underlying mechanisms are similar or not.

Next, we considered whether the reduced gut renewal of *N. ceranae*-infected bees could be the result of ISCs infection. We sectioned midgut tissues to localize parasites at different times

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post-inoculation using FISH and immunostaining techniques and found that stem cells were never infected (Figure 3), even when different proliferative stages were visible in epithelial cells. The absence of ISC infection resulted in a "honey-comb" pattern throughout the entire immunolabelled midgut (Figure S3), arguing for an indirect effect of *N. ceranae* on ISC renewal capacity. Indeed, previous studies have shown that some pathways essential for the control of the intestinal epithelium renewal were perturbed at the transcriptional level after infection. Dussaubat *et al.* (2012) reported that four genes of the Wnt signaling pathway (frizzled2, groucho, basket and armadillo) were down-regulated 10 dpi. This pathway is well known to promote and control the epithelium self-renewal in the *Drosophila* midgut (Lin *et al.*, 2008). Control of gut epithelium renewal is complex and controlled by several signaling pathways that could be divided in two distinctive parts (Figure 4): (i) the EGFR/InR and Wnt pathways which are essential to sustain the basal self-renewal of ISCs under normal conditions and (ii) the Hippo/yorkie, Jak/STAT and JNK pathways which are important in ISC proliferation in response to tissue injury and oxidative stress (Biteau *et al.*, 2011). Hence, we assumed that the parasite could modulate the gut epithelium renewal in a direct (host manipulation by pathogen effectors) or an indirect manner (proliferation perturbation due to tissue damage response). In our experiment, transcriptional analysis by qRT-PCR showed that six genes were significantly upregulated by the infection at 7 dpi (Figure 5). Among them, four were also upregulated at 14 dpi (Keren, Foxo, Ras and Armadillo) and only one gene (Stat) was upregulated at 21 dpi. The expression level of wingless (not shown here) was not significantly different between control and infected honeybees through the entire kinetic. Keren is a cytokine secreted by the enterocyte to sense damage and triggers the proliferation of the ISCs through direct binding to the epidermal growing factor receptor (EGFR) (Ferrandon, 2013). Upregulation suggests that Hippo/yorkie, JNK and/or Jak/STAT pathways may be significantly activated at approximately 7 dpi. This indicates that the infection or the

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damage induced by the infection are detected by the gut and an increase in the epithelium renewal rate should be observed, but is in contradiction with our BrdU labeling results showing a decrease of the replicative activity of the ISCs. This is possibly due to differences in gut renewal regulation in *A. mellifera* or to a disruption elsewhere in the downstream signaling pathways as other pathways also seem to be disturbed by the infection. Except for the Wnt pathway, the different signaling pathways are able to regulate ligands and/or receptors of other pathways, indicating a cross-regulation between pathways (Ammeux et al., 2016). This may explain why our result for the *armadillo* gene is contradictory to Dussaubat et al. (2012) and is evidence that we need to take a holistic approach when studying gut renewal. In this vein, Varelas et al. (2010) reported an evident cross-talk between Hippo and Wnt pathways and that the Hippo pathway may restrict Wnt/β-catenin signaling in human embryonic kidney cells, which may explain the decrease of gut renewal in our experiment; the Hippo pathway may have a larger influence in gut homeostasis, even if both Wnt (the pro-proliferative *Armadillo* gene) and Hippo (the anti-proliferative *hpo* gene) pathways are activated. Moreover, the *foxo* gene involved in the Inr pathway, also upregulated in our study (Figure 4), is known to inhibit proliferation of ISCs (Ferrandon, 2013). It is clearly difficult to identify which pathway is responsible for the gut renewal decrease on the basis of gene expression as regulation of several proteins involved in these pathways occurs through both phosphorylation and protein relocation. New antibodies against honeybee orthologs of the *Drosophila* ligands and receptors involved in gut homeostasis are urgently needed. Using these tools, additional experiments using phosphoproteomic, immunolocalization and functional approaches will be useful to better understand the role of the different signaling pathways in honeybee gut homeostasis.

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In *Drosophila*, apoptosis of enterocytes is triggered during pathogen infection and consecutively leads to an increase in ISC proliferation. Interestingly, blocking the apoptosis reduces the ISC proliferation, even in presence of pathogens (Apidianakis *et al.*, 2009). Hence, another possible explanation for the decrease of ISC proliferation in our experiments could be associated with an inhibition of apoptosis induced by the parasite; several studies have linked *N. ceranae* to disruption of apoptosis (Dussaubat *et al.*, 2012; Holt *et al.*, 2013; Aufauvre *et al.*, 2014; Martín-Hernández et al., 2017). *N. ceranae* is able to reduce apoptosis in infected intestinal cells, possibly to promote its proliferation in susceptiblehoneybees (Higes *et al.*, 2013; Kurze *et al.*, 2015; Martín-Hernández et al., 2017). This could therefore lead to the ISC renewal impairment we observed in our experiment using BrdU labelling, contrary to the work of Martín-Hernández et al. (2017), in which the authors have observed that genes involved in the G1/S phase transition were up-regulated in *Nosema*-infected honeybees, which may indicate a promotion of cell proliferation. But as previously discussed, the gut renewal is also regulated through both post-translational modifications of regulatory proteins and their relocation, possibly explaining the discrepancy between our study and the work of Martín-Hernández et al. (2017).

To conclude, we have shown that *N. ceranae* can negatively alter the gut epithelium renewal rate and disrupt some signaling pathways involved in gut homeostasis. Considering the oxidative burst (Paris *et al.*, 2017) and the higher energetic demand induced by infection (Vidau *et al.*, 2014), alteration of gut renewal could be an another cause of the reduced longevity observed in infected honeybees, as the *N. ceranae*-induced damage does not appear to be compensated by the gut renewal. However, underlying mechanisms are still unknown and further studies are needed for a better understanding of such mechanisms that severely alter the honeybee physiology.

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IV. Acknowledgements

V. Bibliography

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Figure 1. Effect of *Nosema ceranae* infection on honeybee survival. Cumulative proportion of surviving honeybees in both control (black) and N. ceranae-infected bees (red), from day 0 to day 21. Sampling points are represented by vertical dash lines.

Figure 2. Intestinal Stem Cell (ISC) proliferation assay. A) Histogram showing the active crypts/total crypts ratio for both controlled and infected conditions at different times post infection. Significant differences are indicated by asterisks. B) Section of a BrdU-labelled midgut. The honey bee midgut is organized in crypts where stem cells (black arrows) are localized at the basal side. Nuclei of active stem cells appear labelled black after BrdU labelling.

Figure 3. Absence of *Nosema ceranae* infection in intestinal stem cells. Midgut sections (A, B) stained with a Cy3-labelled *N. ceranae* specific probe (FISH staining with Cy3-labelled *N. ceranae* small subunit RNA probe (Nc01: 5'-TACCCGTCACAGCCTTGT-3'), orange, panel A) or with anti-PTP2 polyclonalNcPTP2 antibodies (immunostaining, green, panel B). Midgut sections were also counterstained with DAPI to detect both host and parasite nuclei (blue, panels A and B). At 6 days post-infection, stem cells (arrowhead) appear free of parasites, while proliferative stages (M: meronts, S: sporonts, sporoblasts, evidenced by FISH staining) and spores (S, revealed by DAPI staining) are visible in epithelial cells (panel A). At 10 days post-infection, parasites (stained with anti-PTP2 antibodies) are also absent from stem cells (panel B). Panel C: Summary scheme of infection of midgut cells by *N. ceranae*.

Figure 4. Signaling   pathways involved in the control of the intestinal stem cell proliferation. ISC proliferation rate is the result of the integration of numerous signaling pathways that allow the cells to adapt their proliferation to tissue requirements. Wnt, Inr and EGFR signaling pathways are required for homeostatic proliferation, and Yorkie, JAK-STAT, Nrf2 pathways are required for stress- and injury-induced ISC proliferation response. The deregulated genes analyzed in our study are indicated in green. EC: epithelial cell, ISC: intestinal stem cell. Krn: keren, β-catenin: armadillo, HPO: hippo.

Figure 5. Comparative gene expression analysis between control and *N. ceranae*-infected honeybees. Expression levels of 6 genes implicated in the regulation of the epithelium renewal at 1, 3, 7, 14 and 21 days post infection. Significant differences in gene expression level are indicated by asterisks.
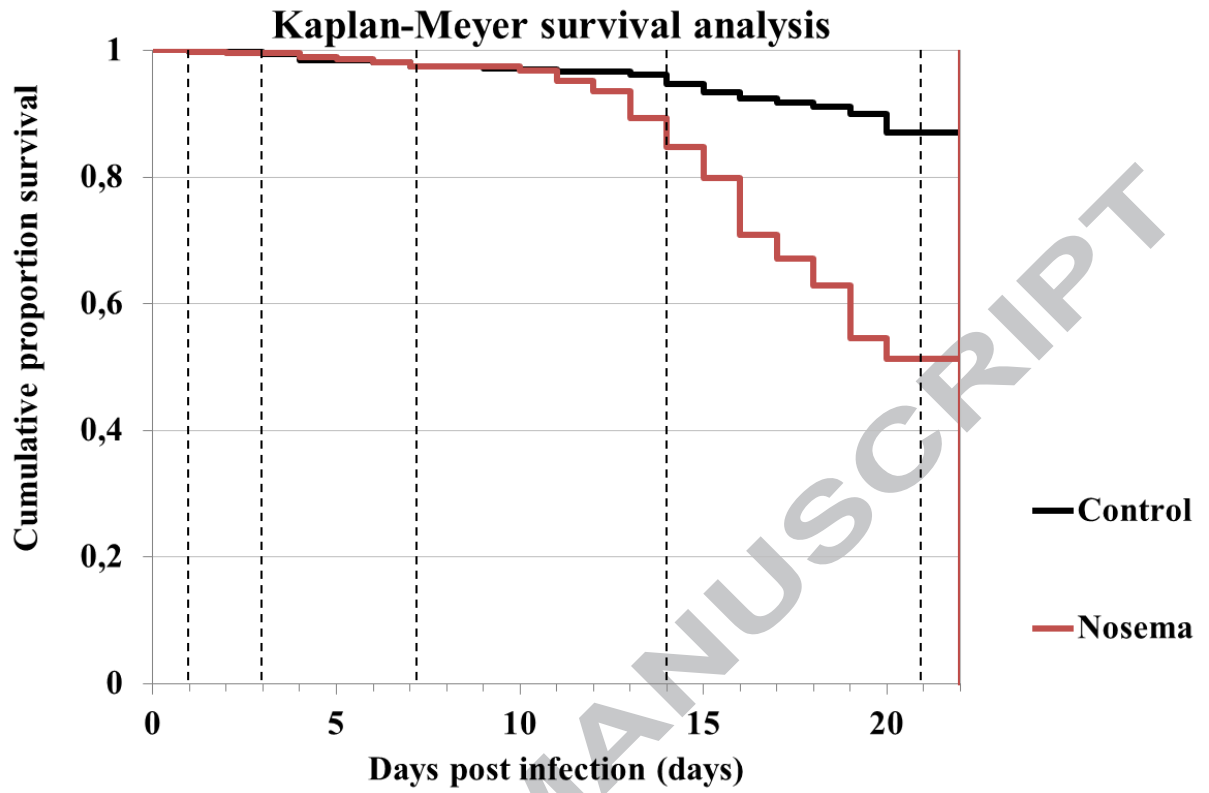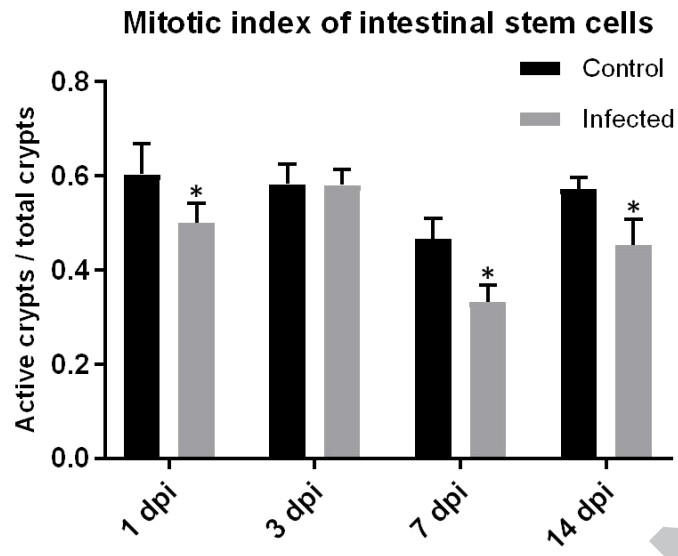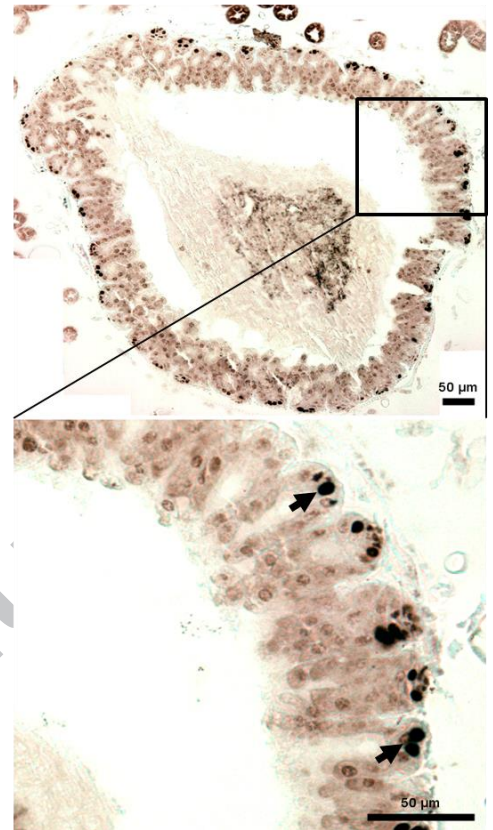
Fig. 1

A)

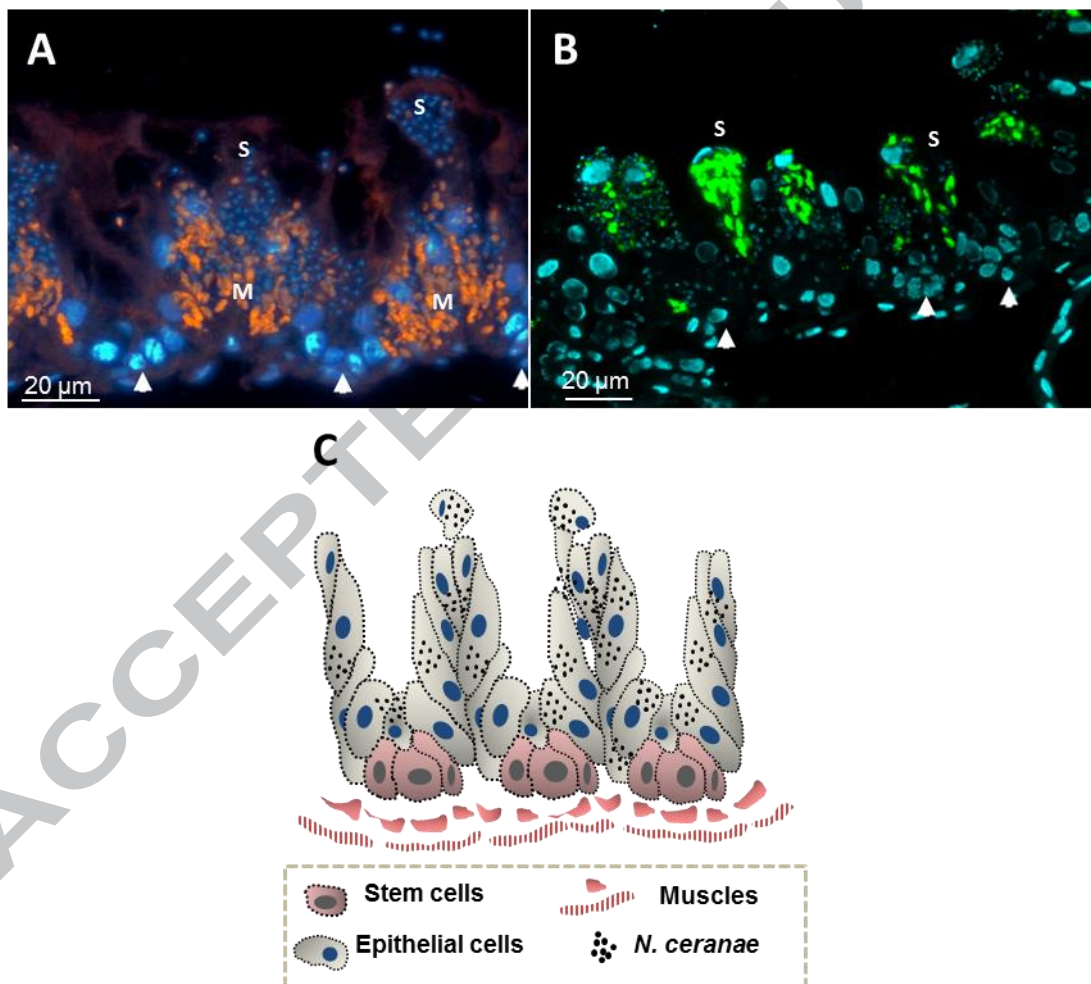B)

Fig. 2

Fig. 3
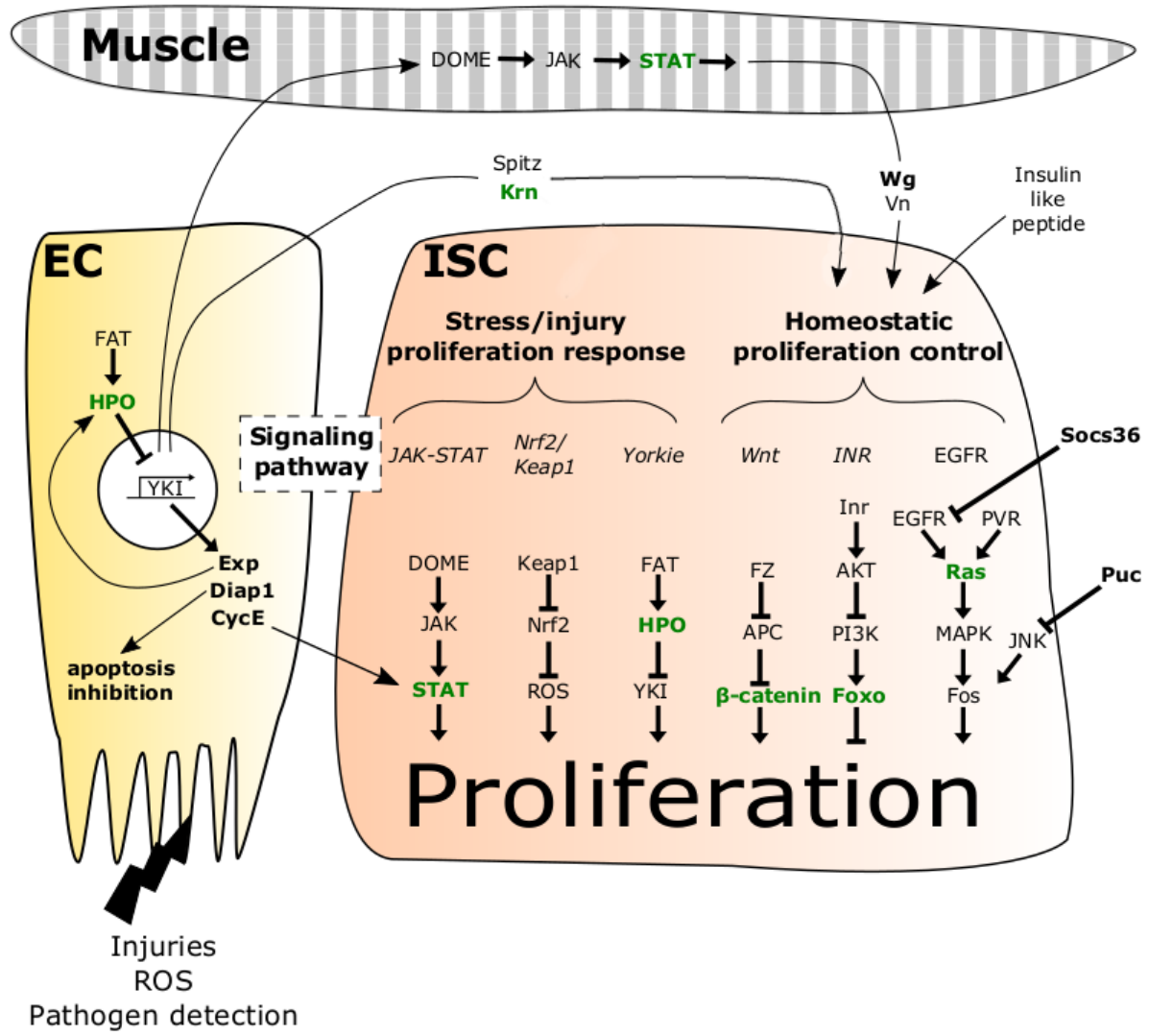
Fig. 4

Fig 5

Graphical abstract

Decrease of the gut renewal capacity

Nosema-infected
honeybee gut

Disruption of signalling pathways
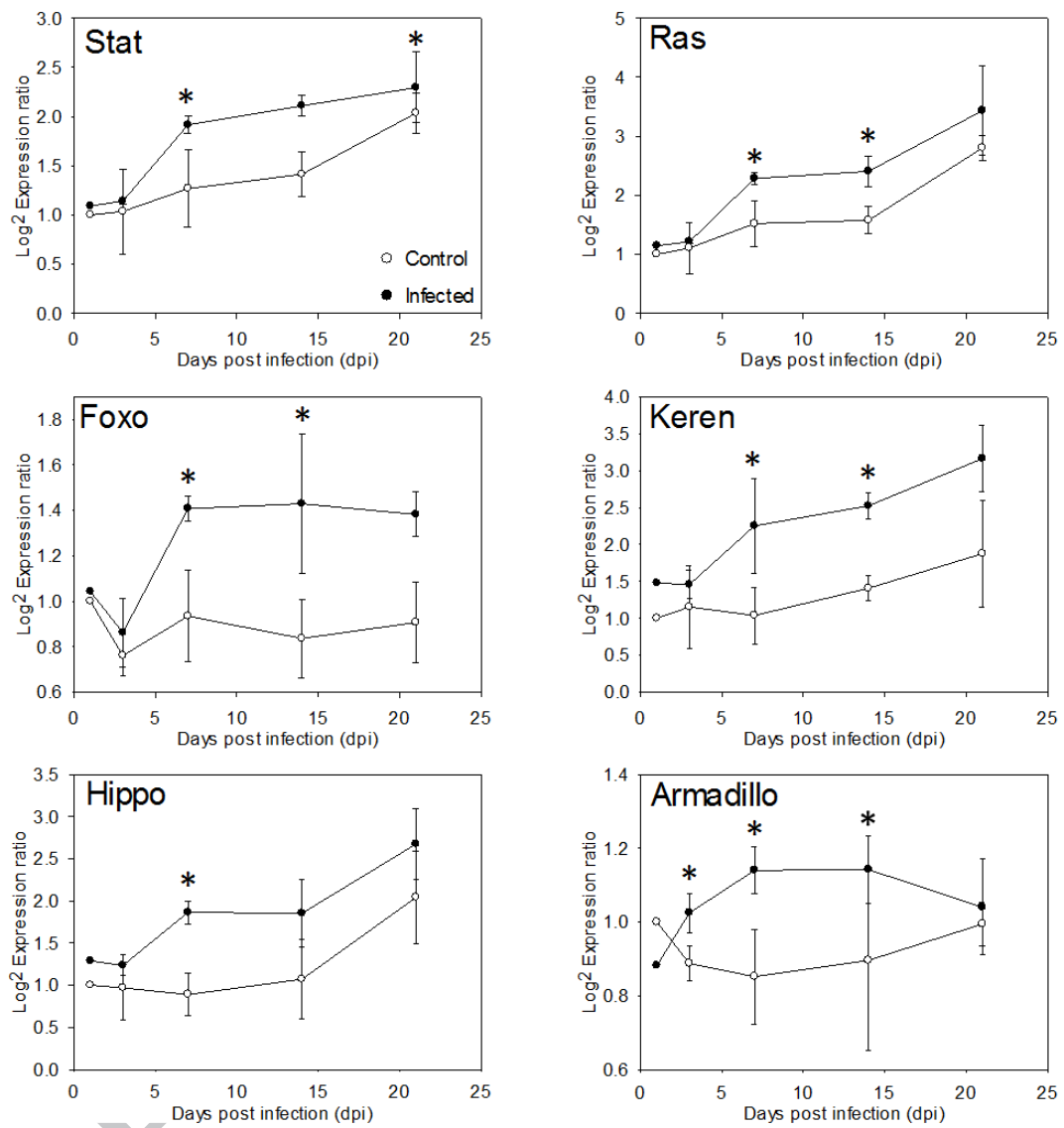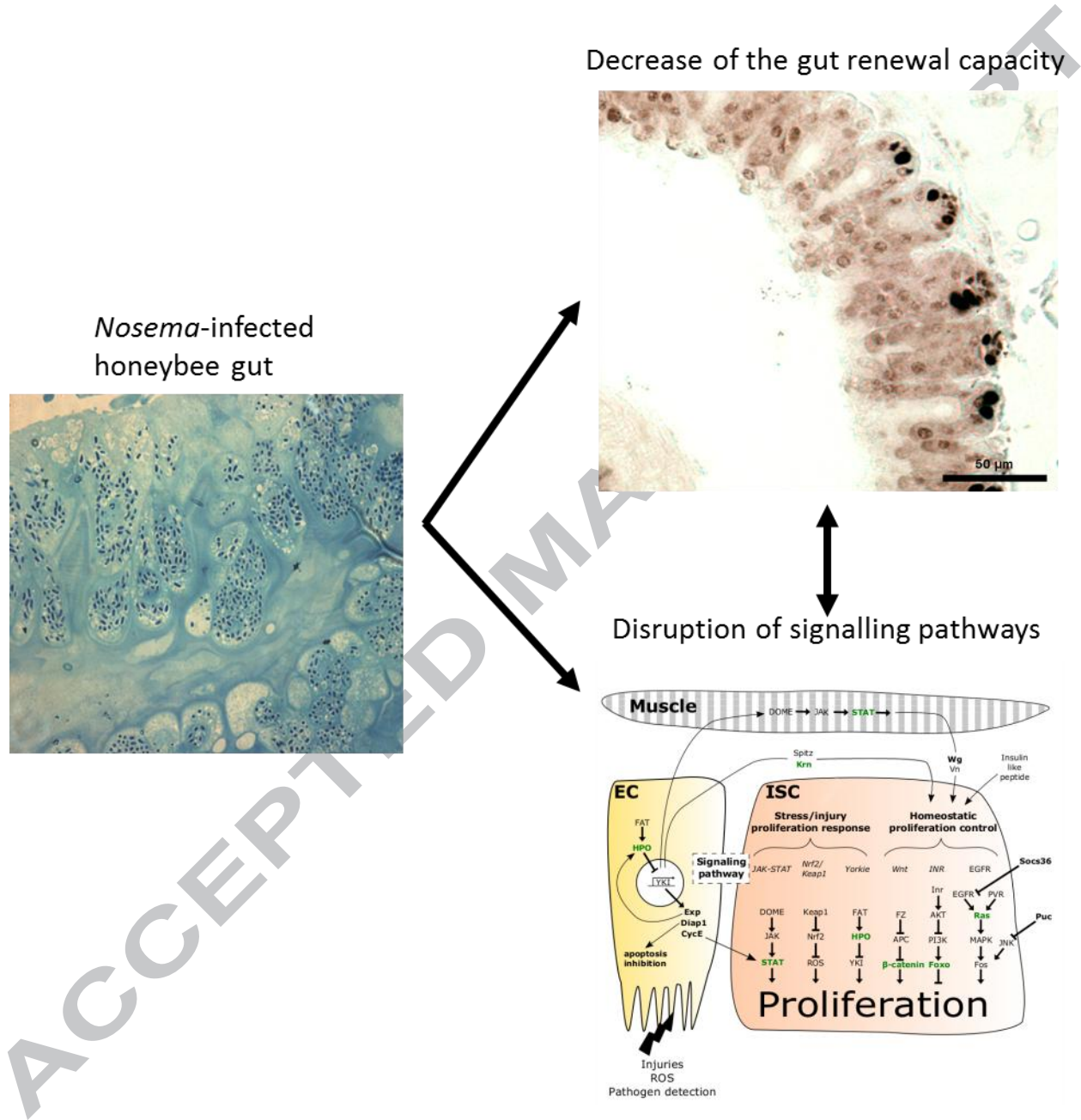
Highlights

► The honeybee midgut homeostasis is disturbed by the microsporidia *Nosema ceranae*.

► Some signaling pathways involved in the gut homeostasis are disruopted.

► Decrease of gut renewal is linked to a reduced bee logevity.