Final Report

Title: Effects of Inducible Reactive Oxygen Species Production on Nosema ceranae Infection

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Introduction

The microsporidian species *Nosema ceranae* and *Nosema apis* can cause individual mortality in honey bees and have been implicated in colony collapse. These obligate intracellular parasites infect the midgut of honey bees, causing energetic stress, epithelial damage, and when untreated, death. Gene expression analysis of infected bees has uncovered a variety of transcriptional changes associated with infection, most notably increases in the transcription of genes involved in protection against damaging reactive oxygen species (ROS).

Our understanding of honey bee immunity is incomplete, especially with regard to mechanisms utilized in the barrier epithelium such as the digestive tract where *Nosema* infection occurs. Studies in other insects have revealed that inducible generation of antimicrobial ROS by epithelial cells is a key component of barrier immunity, and ROS generation has been observed in other insects after infection by microsporidia. However, information is extremely limited on the existence and functional importance of ROS in honey bee immune responses.

In addition to being protective against microbial attack, high levels or chronic exposure to reactive oxygen species is also damaging to host cells and leads to 'oxidative stress'. Bystander oxidative stress in host cells is managed in the short term through host cellular pathways that attempt to reduce ROS levels, repair damage to cellular macromolecules, and initiate tissue level damage responses. If such cellular homeostatic mechanisms are unsuccessful in the long term, cells will undergo programmed cell death, leading to tissue dysfunction, disease, and even death. We have found evidence that bees are considerably more sensitive to oxidative stress than other invertebrate organisms

Hypothesis, and Objectives

We hypothesized that honey bee infection by *N. ceranae* induces ROS production that is insufficient to clear this microsporidian pathogen, but rather results in midgut damage and honey bee death. Our objectives were 1) to determine the effect of infection by *N. ceranae* on ROS production and 2) to examine effects of ROS on *N. ceranae* growth as well as damage to honey bee tissues and honey bee survival.

Results and Discussion

1) Our first objective was to determine effects of *Nosema ceranae* infection on ROS generation. After establishing *Nosema* infection protocols in our lab, we did not find any evidence of increased ROS generation after *N. ceranae* infection over multiple time-points using the Amplex Red Assay, and the ROS detection reagent CM-H2DCFDA. Thus, our data produced as part of the grant from National Honey Board suggests that honey bees do not possess an inducible ROS system that responds to micriosporidian

infection, suggesting that other avenues of research will be critical in understanding the immune response to this pathogen.

We propose three explanations for the results described above. First, it is possible that honey bees do not recognize *N. ceranae* immunologically in a manner that results in ROS production. This would be in contrast to our data showing that other microbes, such as *S. marcescens,* which we have observed to elicit immune ROS production upon ingestion. Second, it is possible that *N. ceranae* inhibits the production of ROS as part of its infection strategy. This hypothesis is testable using co-infections of *Nosema* and *S. marcesens* and will be a focus of future questions. Thirdly, it is possible that assays that are semi-quantitative and utilize whole tissue or even look at whole cells may not be sensitive enough to detect changes in ROS induced by *Nosema* infection. Future efforts will be undertaken to develop more appropriate tools for studying infection at the cell and tissue level.

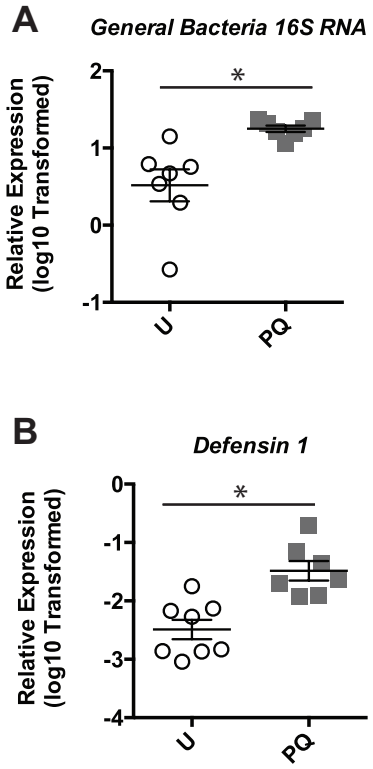

Figure 1. Levels of the general bacteria 16S *(A)* and *Defensin 1* (B) relative to β-actin in midgut tissue from adult bees after 24 hours fed sugar syrup alone or sugar syrup containing Paraquat. Data is log10 Transformed and statistical significance is noted as *p < 0.05.

It is known that *Nosmea* infection caused upregulation antioxidant genes. Part of the initial rationale for the project was that this must be caused by increased ROS. As microsporidia are known to utilize host mitochondria for ATP generation, we then theorized that increased ROS defenses, previously observed in infected bees, could be triggered by increased ROS coming from microsporidia-induced mitochondrial dysfunction. However, we did not find evidence of increased mitochondrial ROS production as assessed by the mitochondrial specific ROS indicator, Mitotracker Red. In addition, we also attempted to examine this issue with the more quantitative assay of mitochondrial genome quantification. However, we did not find reproducible changes in mitochondrial genome equivalents after *N. ceranae* infection, suggesting that the number of functional mitochondria is also not altered.

2) Our second objective was to examine effects of ROS on *N. ceranae* growth as well as damage to honey bee tissues and honey bee survival. As we found no evidence for ROS increases during *Nosema* infection (above), we turned to the model oxidative stress agent Paraquat to examine ROS-induced damage to honey bee tissues. We previously found that Paraquat has a median lethal dose of that is substantially lower than the LD50 dose found in other invertebrate organisms such as the fruitfly, implying that bees are considerably more sensitive to oxidative stress than these organisms. Using Paraquat ingestion, we found evidence that increased ROS produces substantial changes in the midgut. First, we found that Paraquat causes alterations in the microbiome within the midgut, characterized by an increase in

bacterial levels (Figure 1A) and a change in the composition (data not shown). In addition, within this tissue, we find transcriptional up-regulation of immune genes, including those encoding a subset of antimicrobial peptides, such as *Defensin 1* (Figure 1B), as well as select antioxidant genes (data not shown). These results suggest that oxidative stress in the midgut produces substantial effects on both the microbiota resident in the honey bee midgut and immune activation. Future research will focus on the causative relationship between these two phenomena and on understanding how *Nosema* infection might hijack an oxidative response, leading to microbiota and immune gene changes with consequences for midgut immunity and health. In addition, while we did not find that Nosema infection casues increased ROS levels, more studies focused on the effect of ROS on *Nosema* cells are warranted.