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Single and mixed-species trypanosome and microsporidia infections elicit distinct, ephemeral cellular and humoral immune responses in honey bees

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ABSTRACT

Frequently encountered parasite species impart strong selective pressures on host immune system evolution and are more apt to concurrently infect the same host, yet molecular impacts in light of this are often overlooked. We have contrasted immune responses in honey bees to two common eukaryotic endoparasites by establishing single and mixed-species infections using the long-associated parasite *Crithidia mellificae* and the emergent parasite *Nosema ceranae*. Quantitative polymerase chain reaction was used to screen host immune gene expression at 9 time points post inoculation. Systemic responses in abdomens during early stages of parasite establishment revealed conserved receptor (Down syndrome cell adhesion molecule, *Dscam* and nimrod C1, *nimC1*), signaling (*MyD88* and *Imd*) and antimicrobial peptide (AMP) effector (*Defensin 2*) responses. Late, established infections were distinct with a refined 2 AMP response to *C. mellificae* that contrasted starkly with a 5 AMP response to *N. ceranae*. Mixed species infections induced a moderate 3 AMPs. Transcription in gut tissues highlighted important local roles for *Dscam* toward both parasites and Imd signaling toward *N. ceranae*. At both systemic and local levels *Dscam*, *MyD88* and *Imd* transcription was consistently correlated based on clustering analysis. Significant gene suppression occurred in two cases from midgut to ileum tissue: *Dscam* was lowered during mixed infections compared to *N. ceranae* infections and both *C. mellificae* and mixed infections had reduced *nimC1* transcription compared to uninfected controls. We show that honey bees rapidly mount complex immune responses to both *Nosema* and *Crithidia* that are dynamic over time and that mixed-species infections significantly alter local and systemic immune gene transcription.

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1. Introduction

Commonly encountered parasites play an important role in the evolutionary history of a species by imparting strong selective pressures on the development of the immune system, the efficiency of which can impact overall fitness of the species (Schmid-Hempel, 2005). The mechanisms developed and employed by a species to efficiently detect and clear or regulate these infections in their population may vary among individuals depending on a complex combination of host-parasite genetics, parasite dose, symbiont populations, nutrition, and environmental components (Lazzaro and Little, 2009). It is not unusual for multiple parasite genotypes, either distinct strains of the same species or different species, to establish within a single host (Read and Taylor, 2001; Rigaud et al., 2010), further affecting the outcome of infections. Adaptive immune systems of vertebrates are able to develop highly specific responses to complex parasite assemblages via somatic recombination of immune receptors in lymphocyte populations that become increasingly efficient during the course of infection. Although insects lack homologous receptors and cells, they are able to discriminate molecular patterns from particular groups of microbes including Gram positive bacteria, Gram negative bacteria, yeast, fungi and viruses. Novel applications of recognition receptors, including complex receptors on hemocytes that undergo recombination in response to parasite infections like Down syndrome cell adhesion molecule (*Dscam*) (Dong et al., 2012), may enable higher levels of immune regulation and microbial-specific detection (Loker et al., 2004). As a result, unique combinations of effector molecules can be deployed in response to particular types of microorganisms (Lemaitre et al., 1997). This ability makes sense from an evolutionary perspective assuming the response mounted is the result of selective pressure toward efficient host regulation of a parasitic infection that minimizes pathogenicity and metabolic costs to the host. Given that parasite assemblages in hosts are often complex (Rigaud et al., 2010), the

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question is raised as to if or how insect immune responses change when they are infected by disparate groups of microorganisms.

European honey bees (*Apis mellifera*) face a diverse pathosphere (Evans and Schwarz, 2011) throughout their lifecycle that includes two prevalent (Runckel et al., 2011; Cornman et al., 2012) enteric parasites of adults, the microsporidian *Nosema ceranae* and the trypanosome *Crithidia mellificae*. Microsporidians (Fungi; Microsporidia) are very common obligate intracellular parasites of insects that exploit their hosts to varying degrees of impact, yet basic information at the molecular level of the dynamics between Microsporidia and their hosts is limited relative to the abundance of these parasites (Troemel, 2011). Adding complexity to the host-parasite relationship, microsporidians will form concurrent infections in insects with other endosymbiont species (Agnew et al., 2003). Such mixed-species infections can alter parasite development and infection intensity (Solter et al., 2002; Pilarska et al., 2006; Tokarev et al., 2011). The impact such mixed-species infections make on host immune responses have yet to be addressed.

Following a recently successful host transition from the Asian honey bee (*Apis cerana*) to the European honey bee, *N. ceranae* rapidly displaced its congener, *Nosema apis*, which has long been associated with *A. mellifera*. (Klee et al., 2007; Paxton et al., 2007; Chen et al., 2009). This emergent pathogen offers an opportunity for monitoring the progress of host-pathogen interaction and impacts on other endosymbionts. Fecal-oral transmission of *N. ceranae* leads to invasion and replication within epithelial cells lining the midgut (ventriculus) that ultimately destroys the cells as new spores are released to infect neighboring cells (Gisder et al., 2011). Typical of microsporidians, *N. ceranae* have highly reduced genomes (Cornman et al., 2009) and likely rely on numerous molecules from their host cells (e.g. ATP) for essential cellular processes. Conflicting reports on the severity of *N. ceranae* infections to honey bee health (Paxton et al., 2007; Higes et al., 2008; Gisder et al., 2010; Forsgren and Fries, 2010) may be due to known factors including environmental conditions (Martín-Hernández et al., 2009; Gisder et al., 2010) and toxins (Alaux et al., 2010), or to the yet untested role of parasite and microbiota interactions (Evans and Schwarz, 2011).

Trypanosomes (Euglenozoa; Kinetoplastida) are another common but diverse group of parasites that infect insects (Merzlyak et al., 2001). Well studied models in bumble bees (*Bombus*) (Imhoof and Schmid-Hempel, 1999; Brown et al., 2003; Schlüns et al., 2010), flies (*Drosophila*, *Phlebotomus*, *Glossina*) (Boulanger et al., 2001, 2004; Hao et al., 2001) and kissing bugs (*Rhodnius*) (Lopez et al., 2003) have shown increased host mortality and behavioral change can result from trypanosome infections. It is also clear that insects induce diverse immune responses to trypanosomes that centrally involve the production of defensin antimicrobial peptides (AMPs) (Boulanger et al., 2006). Honey bees host *C. mellificae*, which is not known to infect host cells intracellularly but rather lives in the lumen of the gut where they can form a contiguous layer over the endothelium (Langridge and McGhee, 1967). *Crithidia* are regionally common in apiaries of the U.S. (van Engelsdorp et al., 2009; Runckel et al., 2011) and a positive correlation with *N. ceranae* prevalence (Runckel et al., 2011) as well as increased abundance in honey bee colonies affected by Colony Collapse Disorder (CCD) (Cornman et al., 2012) hint at interesting dynamics among endosymbionts and implications toward pathogen virulence that have yet to be understood. The molecular impacts of *C. mellificae* on honey bees including immune responses, either on their own or in conjunction with other microbes, are unexplored areas of importance to understand host-pathogen dynamics on honey bee health.

The honey bee genome encodes the expected key components of an innate immune system (Evans et al., 2006; Weinstock et al., 2006). These include microbial recognition receptors like Dscam, peptidoglycan recognition proteins (PGRPs) and nimrod C-type receptors (eater-like), thioester protein (TEP) opsonins, melanization/encapsulation components, AMPs and components of the Toll and Immune deficiency (Imd) signaling pathways. Binding of microbes by circulating recognition molecules (e.g. Gram negative binding proteins, GNBPs) leads to a soluble proteolytic cascade that culminates in cell membrane Toll receptor binding and intracellular signaling. In contrast, Imd signaling is activated directly following binding of microbial epitopes by membrane-bound recognition receptors (e.g. PGRP-L proteins) on responsive cells (fat body, hemocytes, epithelial) (Ferrandon et al., 2007). Once activated, the Toll and Imd intracellular signaling cascades are complex with transcriptional responses regulated by nuclear factor-κβ (NF-κβ) family transcription factors that determine not only AMP effector responses but also the production of Dscam receptor variants (Dong et al., 2012).

AMPs are diverse peptides important for insect host defense, deployed in large quantities following immune activation typically from the insect fat body located in the abdominal cavity, but also produced locally in tissue by gut epithelial cells and hemocytes (Bulet and Stöcklin, 2005). Five well characterized AMPs are encoded within the honey bee genome (Evans et al., 2006) and act as important components of general barrier immunity in the honey bee gut (Jefferson et al., 2013): abaecin, apidaecin, defensin 1, defensin 2, and hymenoptaecin. In general, AMPs directly lyse microbial cells (i.e. defensin) (Brogden, 2005) but can also be cytostatic, inhibiting enzymatic activities necessary for parasite replication (i.e. apidaecin) (Otvos et al., 2000; Li et al., 2006). Variation in AMP response intensity is linked to honey bee genetic background (Decanini et al., 2007) and colonies that maintain high AMP levels effectively reduce disease caused by particular pathogens, but at a cost to colony productivity (Evans and Pettis, 2005). Thus, there is selective pressure for honey bees to have evolved efficient immunoregulatory mechanisms when combating their pathosphere.

To address the important challenge of understanding the metabolic impacts parasitic infections have on honey bees, we contrast two commonly encountered gut parasites, *C. mellificae* and *N. ceranae*, in adult workers. Both single and mixed-species infections were established and monitored over early and late post-infection (p.i.) times for systemic and local cellular, signaling and humoral gene expression. Here we provide the first description of honey bee immune responses to a trypanosome and contrast it with responses to the emergent pathogen *N. ceranae* individually and during synchronous, mixed species infections. In addition, we examine gene transcription using hierarchical clustering analysis to reveal correlated expression of genes involved in honey bee immune response to these parasites.

2. Materials and methods

2.1. Parasites

C. mellificae type strain ATCC 30254 ('30254') (American Type Culture Collection; Manassas, VA) were cultured axenically in Insectagro DS2 media (Cellgro, Mediatech, Inc.; Manassas, VA) supplemented with 2% heat inactivated fetal bovine serum (Cellgro) and 100 IU/mL penicillin – 100 μg/mL streptomycin solution (Cellgro) at 25 °C. After 48–72 h, cultures were pelleted at 1000 rpm at 4 °C for 10 min, decanted and suspended in 1× phosphate buffered saline (PBS). Cell density was quantified on an improved Neubauer hemocytometer using light microscopy at 400× magnification. 2000 cells/μl suspensions were made in 10% sterile sugar water (1:1 v/v) and 90% 1× PBS.

Nosema spores were isolated from midguts (ventriculus) of foraging worker honey bees collected at the U.S.D.A. Bee Research Lab (BRL; Beltsville, MD). Spores were suspended in 1xPBS and filtered from larger bee tissue through a 500 μm polyester membrane via