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Short Communication

A fluorescent method for visualization of *Nosema* infection in whole-mount honey bee tissues

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ABSTRACT

Honey bees are critical pollinators in both agricultural and ecological settings. The *Nosema* species, *ceranae* and *apis*, are microsporidian parasites that are pathogenic to honey bees. While current methods for detecting *Nosema* infection have key merits, additional techniques with novel properties for studying the cell biology of *Nosema* infection are highly desirable. We demonstrate that whole-mount staining of honey bee midgut tissue with chitin-binding agent Fluorescent Brightener 28 and DNA dye Propidium Iodide allows for observation of *Nosema* infection in structurally intact tissue, providing a new tool for increasing our understanding of *Nosema* infection at the cellular and tissue level.

1. Introduction

The Western Honey Bee, *Apis mellifera*, provides pollination services of critical importance to humans in both agricultural and ecological settings (Potts et al., 2010). Honey bee colonies have suffered from increased mortality in recent years that is likely caused by a complex set of interacting stresses (Goulson et al., 2015). Among the environmental stressors implicated in honey bee disease, there has been intensifying focus on the role of microbial attack on honey bee health (Evans and Schwarz, 2011). The microsporidian species *Nosema ceranae* and *Nosema apis* can cause individual mortality in honey bees and have been implicated in colony collapse (Fries, 2010). Obligate intracellular parasites, these unicellular eukaryotes infect the midgut of honey bees and cause significant pathology at the individual and colony levels. *N. apis* has been recognized as an important pathogen of honey bees for over a 100 years (Fries, 1993). *Nosema ceranae*, not observed in *Apis mellifera* colonies until the mid 2000s, has quickly become highly prevalent in managed colonies of European honey bees all over the world (Higes et al., 2013b).

N. ceranae spores infect the midgut of honey bees, causing energetic stress epithelial damage, and when untreated, death (Dussaubat et al., 2012; Higes et al., 2007, 2013a; Mayack and Naug, 2009). In addition, infection has been associated with a number of physiological and behavioral changes that likely affect individual contribution to the colony (Alaux et al., 2012; Goblirsch et al., 2013; Lach et al., 2015). Gene expression analysis of the infected bees has uncovered a variety of transcriptional changes associated with infection, most notably alterations in epithelial regeneration and metabolic processes (Aufauvre et al., 2014; Holt et al., 2013). To date, no cellular process or pathway has been categorically implicated in honey bee deaths in these experiments, however unchecked infection results in reduction in colony fitness and survival in a manner influenced by region (Gisder et al., 2010; Higes et al., 2008).

Our understanding of the cell biology of *Nosema* infection, and microsporidian infection in other invertebrates, is incomplete (Weiss and Becnel, 2014). Recent studies in *Caenorhabditis elegans* examining *Nematocida parisii* infection have shown the power of whole-mount tissue preparations for increasing our understanding of the biology of microsporidian infection (Estes et al., 2011; Szumowski et al., 2014; Troemel, 2011). The current methods for detecting and quantifying *Nosema* infection (Fries et al., 2013) include manual spore counting using light microscopy (Cantwell, 1970), quantitative PCR (vanEnglesdorp et al., 2009), enzyme-linked immunosorbent assay (ELISA) (Aronstein et al., 2012), *in situ* hybridization (Gisder et al., 2011), and through the use of DNA dyes (Fenoy et al., 2009; Peng et al., 2013). While all these methods have important advantages, these methods for quantifying *Nosema* infection are either not applicable for whole-mount studies or have never been validated in this context. The establishment of a technique allowing for analysis of spatial infection

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dynamics in whole-mount tissue preparations could therefor provide a powerful instrument for increasing our understanding of the biology of infection at the cellular and tissue level.

Based on previous work using chitin-binding agents to identify microsporidian infection in human clinical samples (van Gool et al., 1993), other model systems of microsporidian infection (Niehus et al., 2014; Szumowski et al., 2014; Yokoyama et al., 1996), and honey bees (Qin et al., 2012), we hypothesized that such dyes might be ideal for visualizing *Nosema* infection in conjunction with other cellular dyes, such as the DNA dye Propidium Iodide (PI). The method described provides a new tool for studies aimed at increasing our understanding of biology of *Nosema* infection at the cellular and tissue level.

2. Materials and methods

2.1. Honey bee tissue collection

Honey bees were collected from outbred colonies consisting of a typical mix of *Apis mellifera* subspecies found in North America at different times during the months of April–October in New York, New York. Only visibly healthy bees were collected and all source colonies were visually inspected for symptoms of common bacterial, fungal, and viral diseases of honey bees. Gut tissue was removed from abdomens and midguts were dissected.

2.2. Whole-mount in situ chitin staining

Honey midguts were fixed for 2 h at room temperature (RT) in 3.2% Paraformaldehyde. Fixative was washed out with PBS + 0.1% Triton X-100 (PBST) at RT. Midguts were incubated with or without 0.001% Fluorescent Brightener 28 (FB28), which is also known as Calcofluor White M2R, overnight at 4 °C in PBST. Samples were washed with PBST at RT. For co-staining with PI, FB28-stained midguts were incubated for 4 h at RT with PBS containing RNAse (final concentration of 100 μg/ml). After washing with PBST at RT, midguts were stained for 20 min at RT in PBST solution containing 3 μM PI. Samples were then washed in PBST at RT and visualized using a NIKON SMZ 1500 dissection scope or a Zeiss LSM 510 Meta confocal microscope. All microscopic images in this manuscript were generated using the same microscope and software settings.

2.3. Spore quantification and staining

To isolate spores, midguts from infected or uninfected bees were individually crushed in 0.5 ml H_2O and spore number was assessed by light microscopy. For spore staining, all steps were carried out as above with whole mount-tissues on lysates from crushed midguts amd visualized using a NIKON Elipse E600FN.

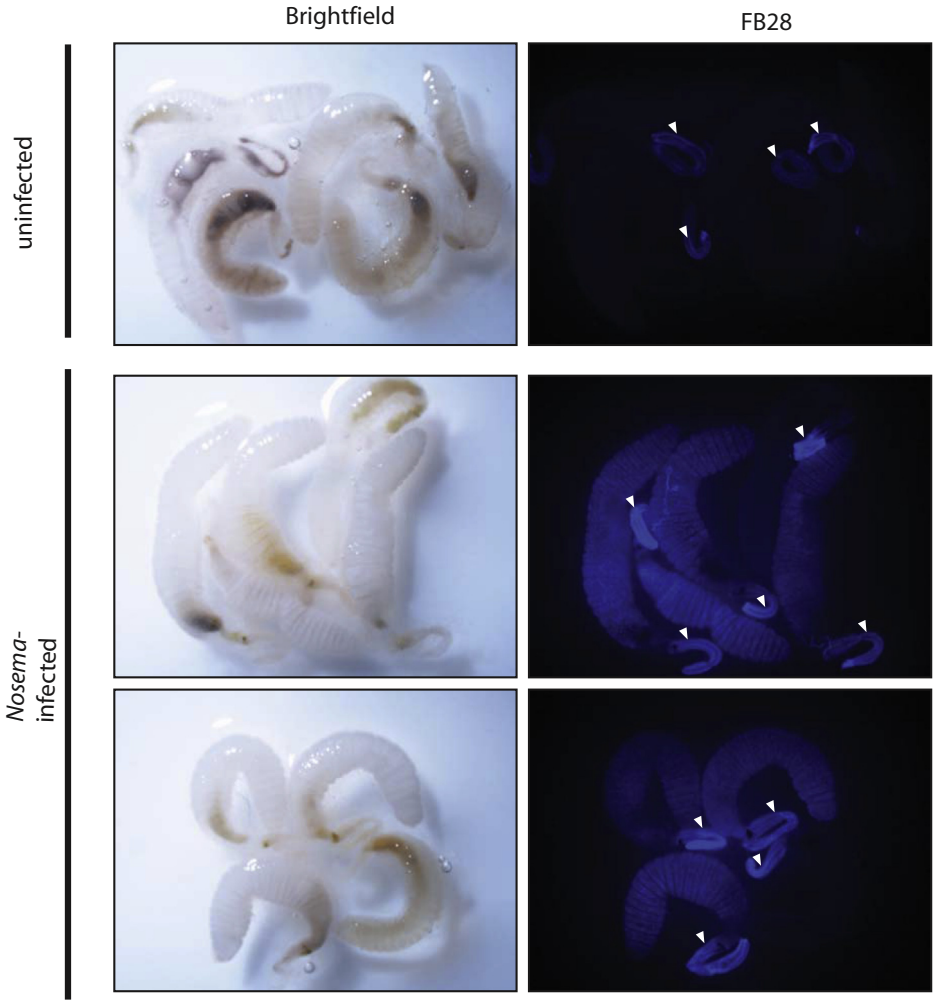

Fig. 1. Chitin-binding dye FB28 allows detection of *N. ceranae* infected midgut regions in whole-mount tissue preparations. Whole-mount midgut preparations from bees from an infected colony and an uninfected colony were treated with FB28 and were visualized using a dissection microscope at 7.5× (peritrophic matrix regions are denoted by white arrowheads).