How honey bees (*Apis mellifera* L.) respond to infection with *Nosema ceranae* and *Lotmaria passim*

by

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A thesis submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

in

Ecology

Department of Biological Sciences

University of Alberta

Abstract

Nosema ceranae and *Lotmaria passim* are two commonly encountered digestive tract parasites of the Western honey bee (*Apis mellifera* L.) that have been associated with colony losses in Canada, the United States, and Europe. Though honey bees can be co-infected with these parasites, little is known about how they affect bee survival, behaviour, or physiology at both the individual and colony level. Using locally-isolated parasite strains, I evaluated the effects of single and co-infections on individual bee survival, responsiveness to sucrose, and the humoral defense response, while at the colony level, I evaluated the effect of these infections on honey bee foraging behaviour and vitellogenin (vg) expression. Results of the survival and sucrose responsiveness experiments showed that infection in general had a significant negative effect on bee survival and also increased bee responsiveness to sucrose, which could correspond to higher levels of hunger and energetic stress. The humoral defense response experiment illustrated that individual bees do not respond locally to infection with *N. ceranae* and *L. passim*, as monitored by the three antimicrobial peptides quantified. At the colony level, I found that at the first instance of foraging, bees that had either single or co-infections had significantly lower vg expression than uninfected bees, with co-infected bees having the lowest vg expression. I also found that co-infected bees had a significantly younger average foraging age (0.6 days) compared to uninfected bees from the same cohort. Collectively, the results of this thesis indicate that single and co-infections involving *N. ceranae* and *L. passim* can negatively affect individual honey bee lifespan, as well as behaviour and physiology, both at the individual and colony level. Changes in behaviour and physiology at the colony level are of great concern as these changes could result in smaller, less productive colonies, decreased colony survivorship,

and reduced income for beekeepers. Because of this, routine monitoring should be undertaken for *L. passim*, and continue for *N. ceranae,* coupled with investigations of novel parasite management strategies.

Preface

Chapter 1 is a general introduction to the topic, study system, and objectives, and is my original work.

Chapter 2 is an adaptation of MacInnis CI, Luong, LT, Pernal SF (2023) A tale of two parasites: Responses of honey bees infected with *Nosema ceranae* and *Lotmaria passim*. Sci Rep 13, 22515. I conceptualized the study with advice from Dr. Pernal. I collected and analyzed the data, and wrote the original draft of the manuscript with feedback from Dr. Pernal and Dr. Luong. Funding was provided by Agriculture and Agri-Food Canada (Project ID:J-001339), an Alberta Graduate Excellence Scholarship, and Project *Apis m*.-Costco Scholarship.

Chapter 3 is an adaption of MacInnis CI, Luong, LT, Pernal SF (2024) Effects of *Nosema ceranae* and *Lotmaria passim* on antimicrobial peptide expression in honey bees. This manuscript is intended to be published in *Applied and Environmental Microbiology*. I conceptualized the study with advice from Dr. Pernal. I collected and analyzed the data, and wrote the original draft of the manuscript with feedback from Dr. Pernal and Dr. Luong. Funding was provided by Agriculture and Agri-Food Canada (Project ID:J-001339), an Alberta Graduate Excellence Scholarship, and Project *Apis m*.-Costco Scholarship.

Chapter 4 is an adaptation of MacInnis CI, Luong, LT, Pernal SF (2024) Effects of *Nosema ceranae* and *Lotmaria passim* on honey bee foraging behaviour and physiology. This manuscript is currently under review with the *International Journal for Parasitology*. I conceptualized the study with advice from Dr. Pernal. I collected and analyzed the data, and wrote the original

draft of the manuscript with feedback from Dr. Pernal and Dr. Luong. Funding was provided by Agriculture and Agri-Food Canada (Project ID:J-001339), an Alberta Graduate Excellence Scholarship, and Project *Apis m*.-Costco Scholarship.

Chapter 5 is a summary and synthesis of the data chapters (2-4), and is my original work.

Dedication

To my mom and dad, Donna and Ian MacInnis. You are my sunshine.

Acknowledgments

I count myself extremely lucky to have known and had the support of so many people for the duration of my PhD and beyond. One of those people is my graduate advisor, Dr. Steve Pernal at Agriculture and Agri-Food Canada (AAFC). I've known Steve for more than 10 years now, and I still feel like I won the advisor lottery. Thank you for taking a chance on me 11(?) years ago, and for meeting with me whenever I asked, despite being busier than what most people would consider humanly possible. Most of all, thank you for all of the opportunities, if I have any success in bee research, it is because of you. Thanks also go to Dr. Lien Luong, my graduate advisor at University of Alberta, for always including me in her lab group, and to Dr. Olav Rueppell for agreeing to serve on my committee.

I conducted all of the fieldwork for my PhD at AAFC's Beaverlodge Research Farm (BRF), and most of the people I've had the pleasure of working with there are more like family than co-workers. I might not be a beekeeper, but Abdullah and Elena always made sure my beekeeping skills were up to snuff. Thank you, Abdullah, for paint marking all the bees I ever needed, and for always getting up early to make nucs or move bees. Most of all, thanks for all the songs!

I am also indebted to the undergraduate students, research assistants, and postdoctoral researchers that have made their way through the Pernal Bee Lab over the years. I would not have been able to inoculate or babysit all the bees used in this thesis without you. A special shout-out to Viviane Beger, who in the summer in 2021 sat outside with me *every day* (rain or 35°C+) counting the number of paint marked foragers returning to their nucs. Thanks also to Patricia Wolf Veiga at the National Bee Diagnostic Center (just up the road from the Pernal Bee

Lab) for teaching me almost everything I know about molecular biology. Your patience is astounding, and I don't know if there is a problem you can't solve!

I am incredibly grateful to all the entities that funded my PhD projects. First and foremost I am grateful to Project *Apis m.* (PAm) who awarded me their Project *Apis m.*-Costco graduate scholarship. This scholarship really allowed me to focus on my research, travel to conferences and pay publication fees without any hassle! Thanks also go to funds received through the Pernal Lab at AAFC, and to the University of Alberta and Government of Alberta for the teaching assistantships, Queen Elizabeth II, and Alberta Graduate Excellence Scholarships.

Doing research for a PhD sometimes gives you imposter syndrome, fills you with grief, and existential dread, and occasionally makes you wonder if what you're doing is worth it. Somehow though, good friends manage to get you through the tough times and to defence. I am grateful for the friends I made while TAing MICRB265, to my friends from the fifth and sixth floors, and to the BRF Squad that always knows when you need a Crooked Creek donut.

Finally, I have to thank the people that got me here, many of whom reside in, or come from northern Ontario. I spent a lot of years watering my grandma's garden, watching birds and butterflies go by while playing cribbage on the deck with my papa, and catching anything I could get my hands on for my "Bug Hut" at the camp with my dad. These experiences are what got me interested in insects, and why I like being outdoors. I was born an only child but the Camerons gave me four brothers, an extra set of parents, and have been cheering me on in all my endeavours for as long as I can remember. To my parents, who encouraged me to follow my dreams, and have always believed I could do whatever I put my mind to (some might use the term 'stubborn' here), I am only here because of you, and I hope reading this makes you proud.

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Chapter 1 Introduction

1.1 General introduction to the honey bee

The Western honey bee (*Apis mellifera* L.) is the world's most intensively managed pollinator required for the pollination of many fruit, vegetable, and high-value cash crops. It is an economically important insect, responsible for producing approximately one third of the world's food crops either directly or indirectly via its pollination services (Klein et al. 2007; Hung et al. 2018). Honey bees contribute an estimated $3-7 billion annually to Canadian agriculture (AAFC 2024), and $15 billion annually to U.S. agriculture (Calderone 2012). In Canada, this figure includes the production of North America's supply of hybrid canola seed (AAFC 2024).

1.2 Eusociality and the physiological underpinnings of age-based division of labour

Honey bee colonies are complex networks comprised of individuals that interact with each other, their environment, and other organisms including parasites both in and outside of the colony. Honey bees possess the three traits that define eusociality: they have overlapping generations, exhibit cooperative brood care, and are divided into castes (reproductive and non-reproductives) (Queller and Strassmann 2003). Individuals within the colony are the offspring of a single female reproductive (queen) that can lay more than 1,200 eggs per day, which often results in a population of 40,000-60,000 individuals per colony living within a small cavity at high density (Winston 1991). The queen's daughters (sterile workers) make up the majority of the population within the colony, and represent the main workforce while her sons (reproductive drones) represent a small portion of the population several times a year (Winston 1991).

As honey bees are eusocial insects, they exhibit age-based division of labour, whereby individuals progress through different tasks within the colony based on their age and physiology (Winston 1991). While plasticity within task performing groups can occur (Huang and Robinson 1992; Robinson 1992; Huang and Robinson 1996; Robinson and Vargo 1997; Toth and Robinson 2005), generally the tasks performed by workers are divided into four broad categories: cell cleaning and capping, nursing and queen care, comb building, cleaning, and food handling, followed by colony ventilation, foraging, and guarding (Winston 1991). Transitioning between tasks is largely physiologically regulated by a feedback loop involving vitellogenin, an egg-yolk precursor protein, and the endocrine factor juvenile hormone (Robinson and Vargo 1997; Amdam and Omholt 2003a; Guidugli et al. 2005; Goblirsch et al. 2013). Workers performing tasks within the colony such as nursing, and nest maintenance have decreased levels of juvenile hormone and increased levels of vitellogenin, and are younger both chronologically and physiologically compared to bees performing riskier tasks outside the colony such as guarding and foraging (Huang et al. 1994; Huang and Robinson 1996; Amdam and Omholt 2003a; Guidugli et al. 2005). As honey bees age chronologically, they also age physiologically whereby decreased synthesis of vitellogenin leads to an increase in the synthesis of juvenile hormone (Amdam and Omholt 2003a; Amdam et al. 2003b). It is this change in physiology that accompanies the transition between tasks in and outside the colony; this has been demonstrated using RNA interference in nurse-aged honey bees, where knockdown of vitellogenin led to an increase in production of juvenile hormone, onset of foraging, and decreased lifespan (Guidugli et al. 2005).

1.3 Honey bee colony losses

Since the mid-2000's, beekeepers have been experiencing levels of winter colony mortality well above the historically acceptable average of 10-15% across Europe, North America, and other regions of the globe (vanEngelsdorp et al. 2007; vanEngelsdorp et al. 2008; Nguyen et al. 2010; van der Zee et al. 2012; Steinhauer et al. 2014; Gray et al. 2020; Oberreiter and Brodschneider 2020; Bruckner et al. 2023; Claing et al. 2023). Canadian beekeepers have reported average winter colony mortality in excess of 20% for the last seven years, and in 2023 reported an average mortality of 32.2%, more than double the acceptable average (Claing et al. 2023). These elevated levels of colony mortality make it difficult for beekeepers to maintain colonies, and underscores the need for the beekeeping industry to improve bee health to ensure crop pollination needs are met (Aizen and Harder 2009).

1.4 Factors contributing to colony losses

Unfortunately, the honey bee is susceptible to a variety of health threats contributing to colony mortality that are difficult to disentangle. These threats include the presence of agrochemicals (Mullin et al. 2010; Wu et al. 2011; Traynor et al. 2016; Gregorc et al. 2018; Walsh et al. 2020), climate change (Ziska et al. 2016), differing management strategies (Oberreiter and Brodschneider 2020; Kulhanek et al. 2021; Steinhauer et al. 2021), poor nutrition (Naug 2009; Chakrabarti et al. 2020; Hoover et al. 2022; Lau et al. 2023), and the presence of a diverse group of parasites and pathogens (Fries et al. 1994; Cox-Foster et al. 2007; Genersch et al. 2010; Evans and Schwarz 2011; Ravoet et al. 2013; Kulhanek et al. 2021; Punko et al. 2021; Borba et al. 2022). Two parasites that have recently been associated with colony mortality are the globally distributed digestive tract parasites *Nosema* (*Vairimorpha*) *ceranae*

(Fries et al. 1996) and *Lotmaria passim* (Schwarz et al. 2015). Though these parasites have been associated with colony mortality individually, they are commonly found concurrently within bees and hives (Runckel et al. 2011; Ravoet et al. 2013; Tritschler et al. 2017; Williams et al. 2021). Despite this, little is known regarding how these concurrent infections affect bees at either the individual or colony level.

1.5 *Nosema ceranae*

Nosema ceranae, recently reclassified as *Vairimorpha ceranae* (Tokarev et al. 2020), is a microsporidian parasite that was first described from the Asian honey bee *Apis cerana* Fabricius in 1996 (Fries et al. 1996). Shortly thereafter, it was determined that *N. ceranae* could infect *A. mellifera* in the laboratory (Fries 1997), and was then subsequently found in managed colonies of *A. mellifera* in 2005, first in Taiwan, and then Spain (Higes et al. 2006; Huang et al. 2007). Historical samples of *A. mellifera* have indicated that this parasite has been present in populations of *A. mellifera* much earlier than 2005 (Klee et al. 2007; Paxton et al. 2007; Chen et al. 2008; Williams et al. 2008a; Invernizzi et al. 2009; Guzmán-Novoa et al. 2011), and has been in the United States since at least 1975 (Traver and Fell 2015) and in Canada since at least 1994 (Currie et al. 2010). *Nosema ceranae* is now considered to be the dominant *Nosema* spp. infecting honey bees, putatively displacing its longstanding congener *N. apis* Zander in regions where both species are present (Chauzat et al. 2007; Paxton et al. 2007; Williams et al. 2008a; Invernizzi et al. 2009; Tapaszti et al. 2009; Currie et al. 2010; Stevanovic et al. 2011; Traver and Fell 2011a; Martín-Hernández et al. 2012; Emsen et al. 2016; Punko et al. 2021). *Nosema ceranae* can infect all adult castes of *A. mellifera* (Fries et al. 1996; Fries 1997; Huang et al. 2007; Alaux et al. 2011; Traver and Fell 2011b), as well as *A. mellifera* worker larvae (Eiri et al.

2015). The parasite has been found in drone *A. mellifera* semen (Roberts et al. 2015), and can infect several other *Apis* spp. (Chaimanee et al. 2010; Botías et al. 2012). The parasite is also cross-infective, capable of causing infection in several *Bombus* spp. (Plischuk et al. 2009; Graystock et al. 2013; Fürst et al. 2014; Gómez-Moracho et al. 2021), stingless bees (Meliponini) (Porrini et al. 2017; Purkiss and Lach 2019), and social wasps (Porrini et al. 2017).

Within *A. mellifera*, *N. ceranae* is described as an obligate intracellular parasite, meaning it relies on its host for its energy supply (see Holt and Grozinger 2016). Honey bees become infected with *N. ceranae* by ingesting spores that germinate within the midgut by everting their polar filaments. If the polar filaments happen to puncture a midgut epithelial cell, sporoplasm is injected and reproduction begins (Fries et al. 1996). Shortly after injection, sporoplasm matures into a meront (beginning of merogony, or reproductive stage of development), and it is this stage of development that is responsible for obtaining energy directly from the host (Holt and Grozinger 2016; Goblirsch 2018)(see Figure 1.1). Meronts possess mitosomes (reduced forms of mitochondria) which allow the parasite to obtain ATP directly from host cells (Holt and Grozinger 2016; Goblirsch 2018). After merogony, sporogony (spore formation stage) begins, and these two stages are delineated by the deposition of electron-dense material onto the plasma membrane of meronts, now referred to as sporonts. Sporonts divide once, producing two sporoblasts, which after polarization occurs, are referred to as spores (Larsson 1986). The epithelial cells filled with these infective spores eventually burst, releasing the spores into the lumen of the gut where they can then go on to be released from the host (e.g., in feces) in order to infect a new host (Cali and Takvorian 2014). Because *N. ceranae* reproduces via binary fission, large numbers of spores can be produced over a short period of time, which can be

detrimental to the host. For example, in cell culture and within bees, *N. ceranae* can fill the cytosol of cells within 3-4 days (Higes et al. 2007; Gisder et al. 2011), and fully developed infections can produce 8×10^6 spores per day (Huang and Solter 2013b).

Within individual honey bees, the negative effects of *N. ceranae* infections are well documented. Infection with *N. ceranae* in adult worker bees degenerates midgut tissues (Dussaubat et al. 2012; Panek et al. 2018), and often leads to reduced lifespan (Higes et al. 2007; Goblirsch et al. 2013; Jack et al. 2016; Arismendi et al. 2020; MacInnis et al. 2023). *Nosema ceranae* can also lead to decreased nursing ability (Goblirsch et al. 2013), and precocious foraging (Mayack and Naug 2009; Goblirsch et al. 2013; Li et al. 2019), as well as increased responsiveness to sucrose (MacInnis et al. 2023). The parasite is also capable of impairing flight (Dussaubat et al. 2013), altering learning and memory (Gage et al. 2018), and affecting the honey bee immune response (Antúnez et al. 2009; Chaimanee et al. 2012; Schwarz and Evans 2013; Li et al. 2018).

The effects of *N. ceranae* at the colony level are much more ambiguous, and seems to vary with climate (Retschnig et al. 2017). In Germany, studies have shown no correlation between *N. ceranae* prevalence and colony mortality (Genersch et al. 2010; Gisder et al. 2010). While in Spain, the presence of *N. ceranae* in colonies has been associated with decreased brood-rearing capacity, colony size, honey production, and increased colony collapse (Higes et al. 2008; Higes et al. 2009; Botías et al. 2013), sometimes without any overt symptoms (Fernández et al. 2012). In the United States, *N. ceranae* was found to be only numerically more prevalent in colonies exhibiting colony collapse disorder (CCD) compared to non-CCD colonies (vanEngelsdorp et al. 2009), while metagenomic analyses illustrated that infection with either *N.*

ceranae or *N. apis* was a differentiating factor between CCD-colonies and healthy colonies (Cox-Foster et al. 2007; vanEngelsdorp et al. 2009). In Canada, increased *N. ceranae* spore abundance in the spring has been associated with increased colony mortality (Punko et al. 2021).

In North America, Fumagilin-B® (DIN: 02231180) is the only registered chemotherapeutic available to treat *N. ceranae* infections (Williams et al. 2008b; Higes et al. 2011; van den Heever et al. 2015a). Unfortunately, this product is not effective against *N. ceranae* in the spore stage, which limits its use in the management of the parasite. Additionally, fumagillin-based products have been on the market since the 1950's in North America, and were originally used to treat infections caused by *N. apis* (Katznelson and Jamieson 1952; Bailey 1953; Peirson and Pernal 2024). At low concentrations, Fumagillin can exacerbate *N. ceranae* infections (Huang et al. 2013a), may be less effective against *N. ceranae* infections compared to *N. apis* infections (Biganski et al. 2024), and commercial formulations which use a dicyclohexylamine salt may have potential toxic side effects in adult worker honey bees (van den Heever et al. 2015b). Because of this, it is imperative that we develop an understanding of how this parasite affects bee physiology, behaviour, and ultimately survival, particularly at the colony level, to determine if beekeepers should continue to monitor for *N. ceranae*. It is also important for the exploration and development of novel management strategies, including the use of: RNA interference technology (Rodríguez-García et al. 2018; Rodríguez-García et al. 2021), porphyrins (Ptaszyńska et al. 2018), genetically engineered bacteria to deliver parasite-specific dsRNA (Lang et al. 2023), pathogen-targeted antibodies (Açık et al. 2024), plant extracts (Porrini et al. 2011a), improvement of honey bee nutrition (Porrini et al. 2011b; Basualdo et al.

2014; Fleming et al. 2015; Jack et al. 2016), and repurposing of beekeeping chemotherapeutics (Parrella et al. 2024).

1.6 *Lotmaria passim*

Lotmaria passim is a recently characterized trypanosomatid parasite that was first described from an *A. mellifera* worker in 2015 (Schwarz et al. 2015). Following its characterization, all trypanosomatid sequences that had previously been accessioned as the trypanosome *Crithidia mellificae* (Langridge and McGhee 1967), a longstanding parasite of the honey bee, were re-evaluated and classified as *L. passim*. This reclassification involved sequences that had been obtained from *A. mellifera* in the United States, Japan, Switzerland, and Belgium, and also included a sequence from *A. cerana* in China (Schwarz et al. 2015), demonstrating the large geographic range of *L. passim*. After its characterization, and the reclassification of *C. mellificae* sequences, *L. passim* was subsequently identified in *A. mellifera* populations in Canada (Borba et al. 2022), Argentina (Castelli et al. 2019),Chile (Arismendi et al. 2016), Italy (Rudelli et al. 2023), New Zealand (Waters 2018), Poland (Michalczyk et al. 2022a), Serbia (Stevanovic et al. 2016), Spain (Buendía-Abad et al. 2021), and Uruguay (Castelli et al. 2019). The parasite can also reportedly infect *A. mellifera* brood (Michalczyk et al. 2022a), and has been present in populations of *A. mellifera* since at least 2006 (Quintana et al. 2021). *Lotmaria passim* is also cross-infective, having been identified in *Bombus pascuorum* Scopoli (Michalczyk and Sokół 2022b), *Bombus terrestris* L. (Bartolomé et al. 2018), and Africanized honey bees (Castelli et al. 2019).

In *A. mellifera, L. passim* is described as an obligate, extracellular parasite of the digestive tract with a strong preference for the distal potion of the ileum, and anterior portion

of the rectum near the papillae in the hindgut (Schwarz et al. 2015; Buendía-Abad et al. 2022). Here, they can obtain nutrients, differentiate into various life stages, and reproduce. Genes related to carbohydrate metabolism were found to be enriched in the *L. passim* genome (Runckel et al. 2014), suggesting that the parasite may be well-adapted to using carbohydrates as an energy source. Four life stages have been described for *L. passim* both in honey bees and under cell culture conditions. These life stages are: spheroid (Schwarz et al. 2015), flagellated (Schwarz et al. 2015; Gómez-Moracho et al. 2020), promastigote (Schwarz et al. 2015), and haptomonad (Buendía-Abad et al. 2022)(see Figure 1.2). The process of differentiation between these stages is likely triggered by changing environmental conditions such as nutritional starvation (Vickerman 1973; Logan et al. 2005). Cytokinesis has been observed for the haptomonad stage of *L. passim* in the honey bee rectum (Buendía-Abad et al. 2022) and in cell culture (Schwarz et al. 2015), though evidence for sexual reproduction in other trypanosomes exists (Gutiérrez-Corbo et al. 2021; Peacock et al. 2021).

The effects of *L. passim* infections on honey bees at the individual level are not well described due to the organism's recent characterization. However, what is currently described generally suggests that *L. passim* has a negative effect on individual honey bee health. There are conflicting reports regarding the parasite's effect on honey bee lifespan, as several studies have shown that infection can negatively affect the duration of life (Liu et al. 2019; Gómez-Moracho et al. 2020; Liu et al. 2020; Buendía-Abad et al. 2021; MacInnis et al. 2023), while one study reports no effect (Arismendi et al. 2020). Individual honey bees exposed to the agricultural pesticide imidacloprid (neonicotinoid) can experience an increase in abundance of *L. passim* compared with unexposed bees (Erban et al. 2023). There is also evidence that honey

bees with atypical microbiomes are more susceptible to infection with *L. passim* than bees with a normal microbiome (Schwarz et al. 2016). Additionally, the parasite can stimulate the honey bee immune system, reduce honey bee nutritional status (Liu et al. 2020), and increase honey bee responsiveness to sucrose (MacInnis et al. 2023). In all of these situations, increased abundance and susceptibility to *L. passim* have the potential to negatively impact honey bee lifespan; this has been verified in several studies where bees have been infected with the parasite (Liu et al. 2019; Gómez-Moracho et al. 2020; Liu et al. 2020; Buendía-Abad et al. 2021; MacInnis et al. 2023).

The way in which *L. passim* affects honey bee colony health is also not well understood given the organism's recent characterization. In one study, *L. passim* was correlated with increased winter colony mortality (Ravoet et al. 2013). Because of the paucity of information surrounding the effects of *L. passim* on individual honey bees and colonies, there are currently no management or treatment recommendations for the parasite. One study has shown *L. passim* to be susceptible to floral volatile compounds *in vivo* and *in vitro* (Palmer-Young et al. 2022), while another found that increasing temperatures of *L. passim* cultures in the presence of *Lactobacillus* inhibited the growth of *L. passim* (Palmer-Young et al. 2023). Both could act as starting points in the development of novel treatment recommendations for *L. passim*, if required.

Interestingly, *L. passim* has been found concurrently with *N. ceranae* in honey bee colonies in Canada, the United States, Switzerland, and Belgium (Runckel et al. 2011; Ravoet et al. 2013; Tritschler et al. 2017; Borba et al. 2022). Nevertheless, we have few studies detailing the effects of mixed (*N. ceranae* + *L. passim*) infections in honey bees at the individual or colony

level (Arismendi et al. 2020), and none detailing the effects of infection under controlled inoculation conditions. This precludes us from fully understanding the pathology of these infections, which makes it difficult for us to generate novel monitoring or management recommendations for beekeepers.

1.7 Effect of parasites and pathogens on honey bee physiology and behaviour

Parasites have the ability to delay or advance the physiological maturation of their hosts (Beckage and Gelman 2004; Schafellner et al. 2007; Goblirsch et al. 2013; Wang et al. 2023). In honey bees, *N.ceranae* can advance physiological maturation by decreasing levels of vitellogenin and increasing levels of juvenile hormone, which subsequently leads to precocious foraging (Goblirsch et al. 2013). Honey bees infected with *Nosema* spp. also have altered flight activity (Dussaubat et al. 2013; Alaux et al. 2014). Infection with *Nosema* spp. affects the length and duration of honey bee foraging trips, as well the frequency with which they make stops between trips, thus reducing their foraging efficiency (Dussaubat et al. 2013; Alaux et al. 2014; Naug 2014; Dosselli et al. 2016). Infection with deformed wing virus can also alter foraging behaviour by reducing flight duration, in addition to the overall distance traveled (Wells et al. 2016). Honey bee colonies also adjust their foraging preferences (sugar content in nectar, lipid and protein content in pollen) in relation to the viral infection status of the colony (Penn et al. 2022).

1.8 Defenses of honey bees against infections

Because honey bee colonies are comprised of related individuals living in small cavities at high densities, they have a number of defenses, both social and individual, that can be used to defend against parasites they constantly encounter (Cremer et al. 2007). Social immunity is the

term used to describe the defense mechanisms that reduce parasite infection intensity and transmission at the colony level, and that confers some benefit to the entire colony (Cremer et al. 2007). These mechanisms function across a gradient, from constitutive to inducible (Simone-Finstrom 2017; Cremer et al. 2018). Constitutive defense mechanisms include polyandry (mating of a queen with multiple males), the transfer of antimicrobial compounds and microbiota, propolis use and task allocation. Inducible defenses include allogrooming (grooming of nestmates), hygienic behaviour (detection and removal of infected brood), behavioural fever (increasing temperature to inhibit the development of symptoms caused by invading organisms), and absconding (leaving the nest, brood, and food behind to find a new nest cavity) (Spivak and Reuter 1998; Boecking and Spivak 1999; Arathi et al. 2000; Spivak and Reuter 2001; Ibrahim and Spivak 2006; Cremer et al. 2007; Borba et al. 2015; Simone-Finstrom 2017; Simone-Finstrom et al. 2017; Goblirsch et al. 2020).

Individual honey bees, like most other insects, also possess a number of defense mechanisms that can be used to protect them against parasites. These include mechanical defenses such as the cuticle and its associated symbionts (*e.g.,* venom peptides), as well as the protective peritrophic matrix lining the gut (Moret and Moreau 2012; Bruner-Montero et al. 2021; de Oliveira et al. 2022; Hong et al. 2022). If parasites are able to breach mechanical defenses, they then become exposed to the innate immune system, which is frequently described as having cellular and humoral components, though they are highly interconnected (Hoffmann 1995; Strand 2008; Zhang et al. 2021). The cellular immune response is mediated by hemocytes and involves responses such as phagocytosis, nodulation, and encapsulation (Strand 2008; Browne et al. 2013; Zhang et al. 2021). Phagocytosis occurs when a hemocyte encounters

a small invader such as bacteria, yeast, or even synthetic beads, and subsequently engulfs it (Strand 2008). Nodulation involves multiple hemocytes binding to clusters of invaders, like bacteria, while encapsulation occurs when invaders, like parasitoids or protozoa, that are too large to be phagocytosed, become surrounded by hemocytes (Strand 2008; Browne et al. 2013). These processes can also be involved in the humoral defense response (Zhang et al. 2021).

The insect humoral defense response is comprised of signalling pathways that mediate responses to invaders (Browne et al. 2013; Zhang et al. 2021). The Toll and IMD (immune deficiency) signalling pathways are the two classic immune signalling pathways that mediate the synthesis of most antimicrobial peptides (AMPs) in response to invading organisms (Hillyer 2016). The Toll and IMD pathways are highly conserved in holometabolous insects, and are present in honey bees (Evans et al. 2006; Nishide et al. 2019). Interestingly, although honey bees have maintained the known immune signalling pathways, they have done so with far fewer components than most other insects, which may be due to the conferred benefits of social immunity (Evans et al. 2006).

Recognition of invaders via the binding of pathogen-associated pattern recognition receptors can activate these signalling pathways, inducing the production and release of compounds with antimicrobial activity, including AMPs (Evans et al. 2006; Danihlík et al. 2015; Hillyer 2016; Zhang et al. 2021), typically from the fat body, but also locally in tissues by gut epithelial cells, hemocytes, and malpighian tubules (Bulet and Stöcklin 2005; Strand 2008; Danihlík et al. 2015; Khan and Han 2024). Honey bees possess five well characterized AMPs from four different families (abaecin, apidaecin, defensins, and hymenoptaein) (Evans et al. 2006). The honey bee-associated AMPs show broad-spectrum activity against bacteria,

protozoa, and fungi, while some may preferentially target one type of invader over another (Danihlík et al. 2015). For example, apidaecin and hymenoptaecin are highly active against Gram-negative bacteria (Casteels et al. 1989; Casteels et al. 1993) while abaecin is slightly less effective against Gram-negative bacteria than apidaecin (Casteels et al. 1993).

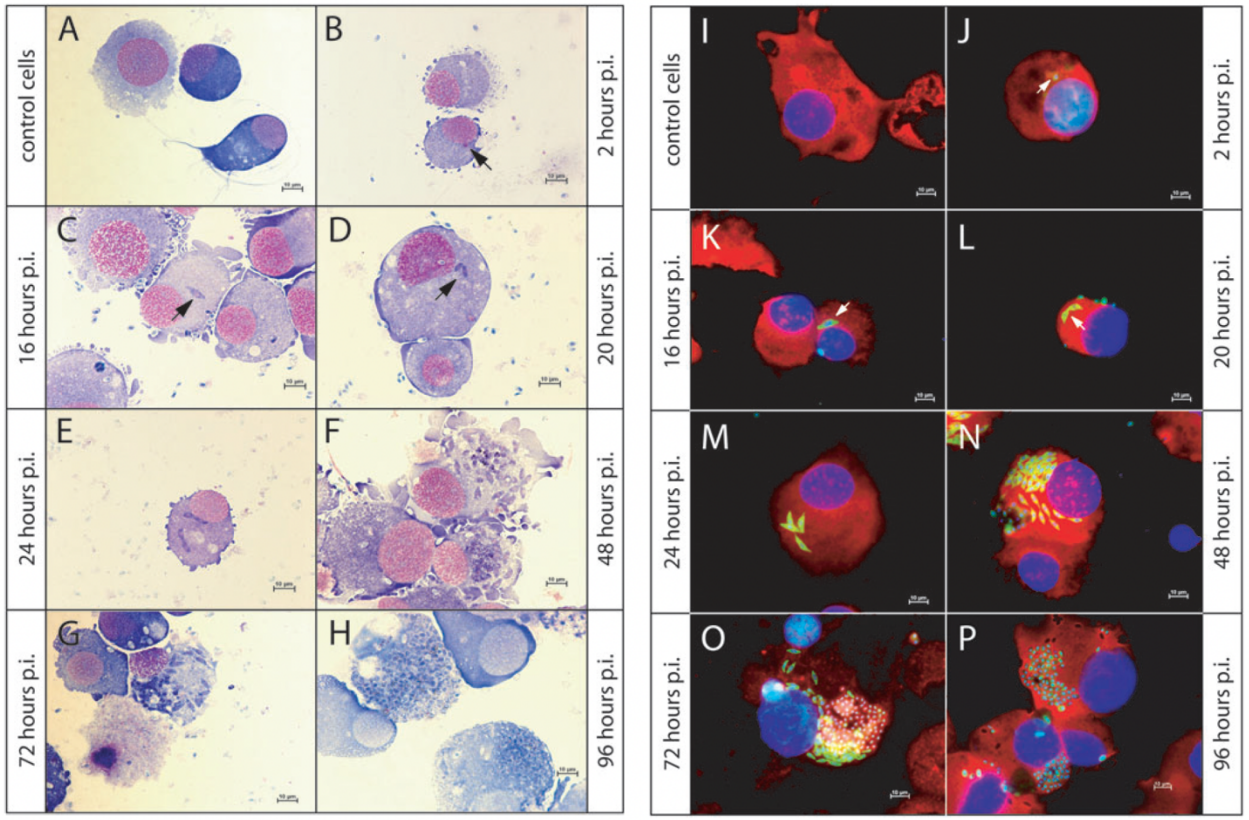
Humoral defenses that honey bees employ in response to invaders are ephemeral and sometimes distinct. A successful infection for *N. ceranae* depends on it breaching mechanical barriers to gain entrance into a gut epithelial cell. In controlled infection experiments, several hours after infecting honey bees with this parasite, significant increases in AMP expression are observed (Schwarz and Evans 2013), followed by a reduction in AMP expression in the days after infection (Antúnez et al. 2009; Chaimanee et al. 2012; Schwarz and Evans 2013; Li et al. 2018). A positive correlation between deformed wing virus and apidaecin expression has also been documented (Jefferson et al. 2013). Evaluating single and mixed *N. ceranae* and *Crithidia mellificae* (Langridge and McGhee 1967) infections further illustrates that honey bees elicit distinct and ephemeral responses to invaders, as studies have shown both changes in individual AMP expression over time, as well as variation in the type of AMPs used in response to different combinations of infections (Schwarz and Evans 2013). For example, for infections involving only *N. ceranae*, honey bees expressed five AMPs, in contrast with single *C. mellificae* and mixed *N. ceranae* and *C. mellificae* infections, which resulted in the expression of two and three AMPs respectively (Schwarz and Evans 2013).

1.9 Thesis objectives

Given the lack of information surrounding infections involving *L. passim*, (particularly mixed *N. ceranae* + *L. passim* infections), the goal of this thesis is to develop a comparative understanding of how single and mixed *N. ceranae* and *L. passim* infections affect honey bee survival, behaviour, and physiology at the individual and colony level in an effort to determine if *L. passim* requires monitoring by the beekeeping community, and if novel parasite management strategies should be developed. Therefore, the specific goals for this thesis were to:

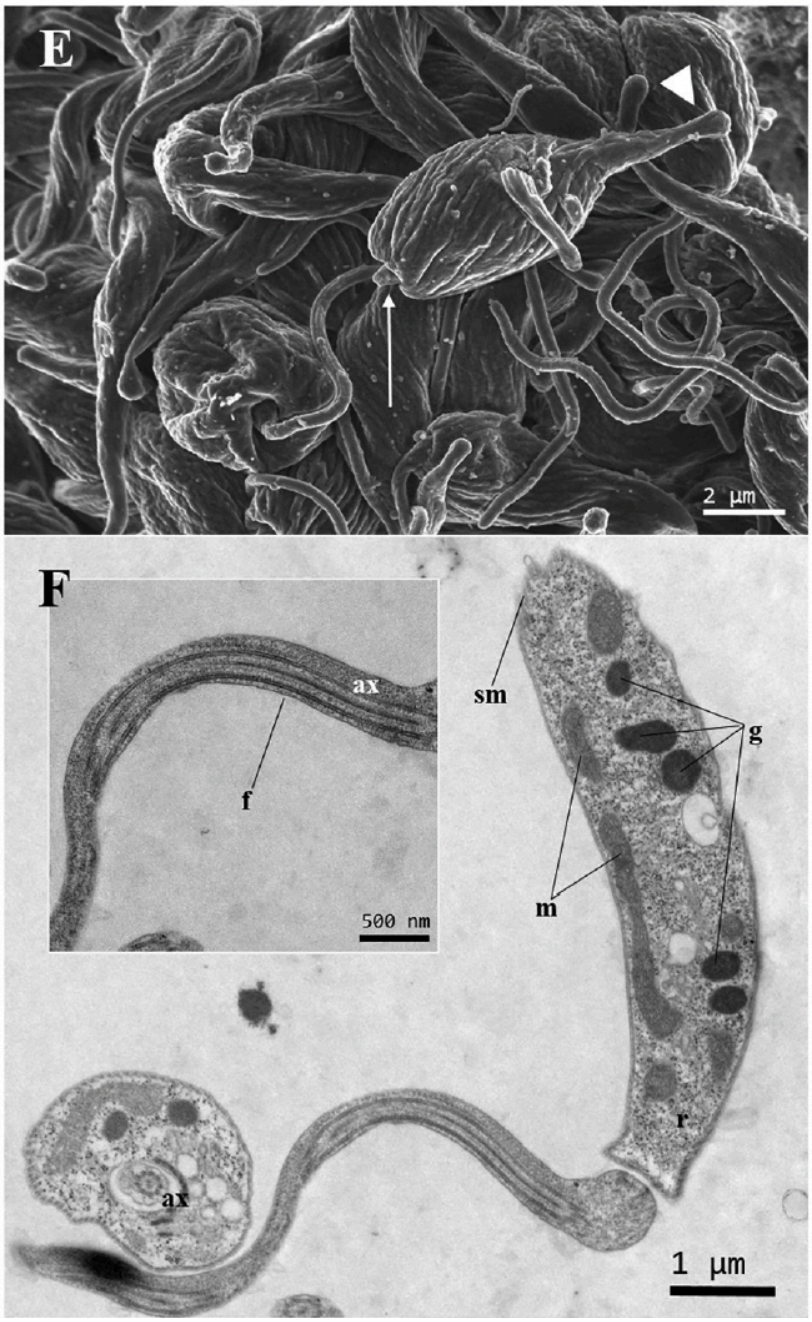
1. Evaluate the effect of single and mixed *N. ceranae* + *L. passim* infections on individual honey bee lifespan, and their responsiveness to sucrose to determine if infections negatively affect honey bee survival, hunger, and energetic stress.
2. Describe the localized humoral defense response (midgut and hindgut tissues) for individual honey bees infected with single and mixed *N. ceranae* + *L. passim* infections over time, and quantify temporal parasite density to determine if the parasites have a tissue preference.
3. Investigate the effect of single and mixed *N. ceranae* + *L. passim* infections on honey bee physiology by quantifying vitellogenin expression over time, and determining if any changes in physiology (vitellogenin expression) correspond to changes in behaviour (first instance of foraging, average forager age, foraging effort) at the colony level.

1.10 Figures and Tables



[1]**Figure 1.1** Developmental stages of *Nosema ceranae* in IPL-LD-65Y (*Lymantria dispar*) cells viewed with differential imaging contrast, and fluorescent microscopy after Giemsa staining and fluorescent *in situ* hybridization. **B and J)** injected sporoplasm (marked with arrows) **C, D, K, and L)** develops into meronts **F and N)** cells contain high numbers of meronts **F, G, and O)** first sporonts are formed **H and P)** cells filled with spores.

[1] Figure adapted from Gisder, S. *et al.* (2011) A cell culture model for *Nosema ceranae* and *Nosema apis* allows new insights into the life cycle of these important honey bee-pathogenic microsporidia. Env Microbiol 13:404-413



[2]**Figure 1.2** Scanning electron microscopy **(E)** and transmission electron microscopy **(F)** images of *L. passim* promastigotes. **E)** *L. passim* promastigotes in culture **F)** Longitudinal section of a *L. passim* promastigote with the single flagellum.

[2] Figure adapted from Buendía-Abad, M. *et al.* (2022) First description of *Lotmaria passim* and *Crithidia mellificae* haptomonad stages in the honey bee hindgut. Int J Parasitol 52:65-75

Chapter 2: A tale of two parasites: Responses of honey bees infected with *Nosema ceranae* and *Lotmaria passim*

2.1 Introduction

The Western honey bee (*Apis mellifera* L.) is the world's most intensively managed pollinator required for the pollination of many fruit, vegetable, and high-value cash crops. Unfortunately, the health of this insect is plagued by a number of factors, including the presence of a variety of pests and parasites (Evans and Schwarz 2011). The microsporidian *Nosema ceranae* and the trypanosomatid *Lotmaria passim* are two of these parasites. They are two globally-encountered digestive tract parasites of the honey bee (Higes et al. 2006; Runckel et al. 2011; Morimoto et al. 2013; Ravoet et al. 2013; Schwarz et al. 2015; Arismendi et al. 2016; Holt and Grozinger 2016; Goblirsch 2018; Waters 2018). *Nosema ceranae* was first described in 1996 (Fries et al. 1996), almost 90 years after its congener *N. apis* (Zander 1909), and has now largely replaced *N. apis* in most regions where both are present (Chauzat et al. 2007; Paxton et al. 2007; Williams et al. 2008b; Invernizzi et al. 2009; Currie et al. 2010; Stevanovic et al. 2011; Traver and Fell 2011a; Emsen et al. 2016). Similarly, the recently described *L. passim* (Schwarz et al. 2015) has managed to outpace *Crithidia mellificae* (a trypanosomatid described from honey bees more than 50 years earlier) (Langridge and McGhee 1967) in terms of prevalence (Runckel et al. 2011; Ravoet et al. 2013; Schwarz et al. 2015; Arismendi et al. 2016; Tritschler et al. 2017; Waters 2018; Bartolomé et al. 2020).

Despite the cosmopolitan distribution of these two recently described digestive tract parasites (Cornman et al. 2012; Schwarz et al. 2015; Goblirsch 2018) that can co-occur (Runckel et al. 2011; Ravoet et al. 2013; Tritschler et al. 2017), we still lack a full understanding of the comparative effects of single and mixed *L. passim* and *N. ceranae* infections in honey bees. This

is because *L. passim* is likely still underreported due to its emerging status, and it being previously misidentified as *C. mellificae* (Schwarz et al. 2015). The organism has also largely been ignored by researchers due to it being considered benign (Langridge and McGhee 1967), despite it being reported as the most prevalent non-viral parasite in a cross-country study from the U.S.A. (Cornman et al. 2012). It is important to evaluate and understand comparative effects because different parasites and infections (single or mixed) can have varying effects on hosts, which could influence parasite management recommendations. For example, *Varroa destructor* and *Acarapis woodi*, two parasitic mites of the honey bee, synergistically decrease honey bee colony survival when present as dual infestations, even though *A. woodi* is often considered to be inconsequential or variable in impact (Downey and Winston 2001). Due to the highly negative effect of this parasite when present with *V. destructor*, beekeepers were recommended to treat colonies for both parasites, in an era when the former organism was not routinely considered (Downey and Winston 2001).

Within individual honey bees, *Nosema ceranae* infects the midgut epithelial cells (Fries et al. 1996; Goblirsch 2018), can degenerate midgut tissues (Dussaubat et al. 2012; Panek et al. 2018), alter foraging behaviour (Mayack and Naug 2009; Goblirsch et al. 2013), stimulate the immune system (Schwarz and Evans 2013), suppress the immune system (Antúnez et al. 2009; Li et al. 2018), alter learning and memory (Gage et al. 2018), induce energetic stress (Mayack and Naug 2009; Li et al. 2018), and decrease nursing ability and lifespan of infected bees (Higes et al. 2007; Goblirsch et al. 2013). How *N. ceranae* affects honey bee colonies is much less clear, and varies with geographic location. In Spain, colonies infected with *N. ceranae* can experience decreases in colony size, honey production, brood-rearing capacity, and colony collapse (Higes

et al. 2008; Higes et al. 2009; Botías et al. 2013), but can also experience no pathological effects (Fernández et al. 2012). In western Europe, studies have shown no relationship between *N. ceranae* prevalence and colony mortality (Genersch et al. 2010; Gisder et al. 2010). A study conducted in the United States showed that colonies with colony collapse disorder (CCD) had only slightly higher *N. ceranae* prevalence and abundance than control colonies (vanEngelsdorp et al. 2009), while metagenomic analyses showed that infection with both *Nosema* spp. was a differentiating factor between healthy colonies and colonies diagnosed with CCD (Cox-Foster et al. 2007; vanEngelsdorp et al. 2009).

Lotmaria passim is found predominantly in the honey bee hindgut with a strong preference for the anterior rectum near the papillae and the distal portion of the ileum (Schwarz et al. 2015). Though the preferred location of *L. passim* is known, the effects of the parasite on honey bee health are poorly understood. Within individual honey bees, there are conflicting reports regarding the parasite's effect on longevity, as reports have shown both decreased (Gómez-Moracho et al. 2020; Liu et al. 2020; Buendía-Abad et al. 2021) or unaffected (Arismendi et al. 2020) lifespans. At the colony level, *L. passim* has been correlated with increased winter colony mortality (Ravoet et al. 2013), the collapse of colonies (Runckel et al. 2011), and can also be found concurrently with *N. ceranae* (Runckel et al. 2011; Ravoet et al. 2013; Tritschler et al. 2017).

Nosema ceranae increases honey bee energetic stress leading to decreased survival (Panek et al. 2018). We do not know how or if individual *L. passim* or mixed infections of the two parasites affect honey bee energetic stress, but a sucrose responsiveness assay could be used as a proxy, whereby increased responsiveness to sucrose could correspond to higher levels

of hunger and increased energetic stress (Mayack and Naug 2009). The sucrose responsiveness assay involves restrained bees and a series of sucrose solutions that vary in concentration (Page et al. 1998). The antennae of restrained bees are touched with droplets of these sucrose solutions in order of ascending concentration, and when a concentration of sucrose is acceptable, a bee responds by extending her proboscis (Page et al. 1998; Mustard et al. 2012). Honey bees that respond to more concentrations of sucrose have increased responsiveness to sucrose (resulting in higher sucrose response scores [SRS]) compared to those responding to fewer concentrations (Page et al. 1998; Mustard et al. 2012).

Given the ubiquitous reports of *N. ceranae* and/or *L. passim* infections on honey bees, and the negative effects on bee health, an investigation is warranted to determine if novel management strategies are required for infections involving *L. passim*. Here, we investigate the effect of single and mixed *N. ceranae* and *L. passim* infections on individual honey bee survival and responsiveness to sucrose, using locally obtained parasite strains and honey bee stock. We hypothesize that parasitic infection will lead to increased sucrose responsiveness. We predict that honey bees inoculated with both parasites will have shorter lifespans and increased responsiveness to sucrose than those inoculated with either *N. ceranae* or *L. passim* due to the increased density and diversity of parasites, and the geographic separation of the parasites within the digestive tract, suggesting reduced interspecific competition.

2.2 Methods and Materials

2.2.1 Parasites

An axenic culture of *L. passim* isolated from the dissected ileum of an adult honey bee worker at Agriculture and Agri-Food Canada's (AAFC) Beaverlodge Research Farm (55°11'43.0"N; 119 °17'57.3"W) was established in the fall of 2016 (cytochrome b (cytb) gene sequenced to confirm

species, see Supplementary Table 2.1). The culture was grown in a water-jacketed incubator at 25 ± 0.1°C (model 3326, Forma Scientific, Ottawa, ON, Canada) (Schwarz et al. 2015) to high density in Schneider's *Drosophila* medium (Cat# 21720024, Fisher Scientific, Ottawa, ON, Canada), supplemented with 10% fetal bovine serum (Cat# 16140071, Fisher Scientific, Ottawa, ON, Canada), and 100 IU/mL Penicillin-100 μg/mL Streptomycin-2.5 μg/mL Amphotericin B (Cat# 30004CI, Fisher Scientific, Ottawa, ON, Canada). The culture was then cryopreserved in liquid nitrogen, and when needed, thawed, and grown to high density in 15 and 50mL centrifuge tubes at 25 ± 0.1°C in the supplemented Schneider's *Drosophila* medium mentioned above. Prior to inoculation, *L. passim* cultures were centrifuged at 200 × *g* for 10 min. After this initial centrifugation step, the supernatant was removed, and filtered through a 0.22 μm filter, while the pellet was resuspended in 1 mL of 1× phosphate-buffered saline (PBS). The filtered supernatant was centrifuged once at 200 × *g* for 10 min, the resulting supernatant removed, and any remaining pellet resuspended to 1mL with a 1:10 sucrose (50% *w/v*): PBS (1×) solution. The resuspended *L. passim* pellet was successively washed and centrifuged twice in 1 mL of 1× PBS at 200 × *g* for 10 min. After a final resuspension of the pellet in 1mL of 1× PBS, a count was performed using a Helber Z30000 counting chamber (Cat# Z30000, Hawksley, Sussex, UK) to estimate the number of motile, flagellated *L*. *passim* cells/mL of culture.

Nosema ceranae spores were obtained from the dissected midguts of *N. ceranae*-infected adult *A. mellifera* workers at AAFC's Beaverlodge Research Farm; the procedure for spore collection was adapted from MacInnis et al. 2020. After dissection, midguts were manually macerated in 1 mL of 1× PBS in a Stomacher® 80 Biomaster Standard Bag (Cat# BA6040, Seward, West Sussex, UK) for 1 min, before maceration in a Stomacher® 80 blender

(Cat # 030010019, Seward, West Sussex, UK) for 5 min. The macerate was then passed through a 40 μm cell strainer (Cat# 352340, Fisher Scientific, Ottawa, ON, Canada) and rinsed with 15 ml of 1× PBS. The resulting filtrate was then vacuum-filtered through a 10 μm separator (Cat# 60344, Pall Corporation, Ann Arbor, MI, USA) and rinsed with another 15 mL of 1× PBS. The resulting 30 mL filtrate was then centrifuged at 800 × *g* for 10 min, and the pellet resuspended in 1 mL of 1× PBS. The 1 mL of *N. ceranae* spores in 1× PBS was then treated with 100 IU/mL Penicillin-100 μg/mL Streptomycin (Cat# 15140122, Fisher Scientific, Ottawa, ON, Canada) for 1 hr to kill any contaminating bacteria (Schwarz and Evans 2013). The *N. ceranae* spores were then washed 3 times in 1 mL of 1× PBS followed by centrifugation at 800 × *g* for 10 min. After the final resuspension in 1 mL of 1× PBS, a count was performed using a Helber Z30000 counting chamber to estimate the number of spores/mL (Cantwell 1970). *Nosema* spp. were verified via conventional polymerase chain reaction (PCR) techniques outlined in van den Heever et al. (van den Heever et al. 2015b) with the following modifications: 200 μL of macerate were used for DNA extractions; 75 ng of total DNA was amplified via PCR; and primers NoscRNAPol-F2/NoscRNAPol-R2 NosaRNAPol-F2/NosaRNAPol-R2 as well as thermal cycler settings in Gisder and Genersch (2013) were used to differentiate between *N. apis* and *N. ceranae*.

2.2.2 Experimental bees

Frames of eclosing worker bees were collected from nonexperimental colonies managed by the Apiculture Program at AAFC Beaverlodge. Four to six frames from four to six different colonies confirmed to be *Nosema* spp.-free and trypanosomatid-free via PCR were maintained in a 33°C ± 1.0°C programmable incubator (models I36NLC8, I36NLC9, Percival Scientific, Perry, IA, USA) at

any given time. Bees were collected from these frames daily, so that all newly-emerged bees (NEBs) used for experiments were <24 hrs old, and free of any *Nosema* spp. and trypanosomatid spp. infections.

2.2.3 Survival

Individual NEBs were orally-inoculated with 5 μL of one of the five following treatment groups diluted in 1:10 sucrose (50% *w/v*): PBS (1×) solution via a 10 μL pipette: sucrose control (1:10 sucrose:PBS solution only), media control (any resulting pellet from the centrifuged *L. passim* supernatant), *N. ceranae* only (1.0×10^5 *N. ceranae* spores), *L. passim* only (1.2×10^5 motile, flagellated *L. passim* cells), and *N. ceranae* + *L. passim* (1.0×10^5 *N. ceranae* spores + 1.2×10^5 motile, flagellated *L. passim* cells). After inoculation, NEBs were maintained individually in 15 mL centrifuge tubes for 30 min to ensure the inoculum was ingested (no inoculum droplets observed within the centrifuge tubes) before caging occurred. After this 30 min, NEBs that had not fully consumed their inoculum were discarded, while those that did were caged by treatment. Each of the five treatments consisted of two cages (A and B); each containing an average of 51 ± 0.6 inoculated NEBs, along with an average of 48 ± 0.7 uninoculated NEBs (thoraxes paint-marked to identify them) to provide social interaction, and to act as controls that received minimal handling (see Supplementary Table 2.2 for details). Cages (plastic cages used to hold NEBs) were maintained at $33°C \pm 1.0°C$ in programmable incubators, and NEBs were provisioned on 50% *(w/v)* sucrose in a gravity feeder, and pollen patty in a diet tray *ad lib.* The pollen patty was prepared by Global Patties (Airdrie, AB, Canada) according to their standard recipe, but modified to include 25% [by weight] irradiated Canadian-collected *B. napus* pollen. The modified *(w/w)* recipe contained 46% sucrose syrup, 15% distillers dried yeast, 14%

defatted soy flour, and 25% irradiated *B. napus* pollen. Diet was replaced every 72 hrs, and mortality was monitored daily until total mortality occurred for each treatment during the summer of 2018 when all four replicates were conducted. Dead bees were removed from cages as they appeared, and stored at -20°C for further processing. Unfortunately, some total cage mortality occurred that was not due to experimental infection (e.g. clogged sucrose feeders), but each treatment was accounted for in at least three of the four replicates (see Supplementary Table 2.2).

2.2.4 Confirmation of infection

In order to confirm infection, and to ensure that cross-contamination did not occur between treatments, we examined NEBs that were dead at 15 dpi. If no NEBs were dead at 15 dpi, we took dead NEBs at the next time point they occurred (e.g., 17 dpi). We examined 2-4 dead NEBs per treatment group per replicate. We confirmed *N. ceranae* was the only *Nosema* spp. present in our experiment, and only present in treatments that contained *N. ceranae* via endpoint PCR as above (see 2.2.1 *Nosema ceranae*) with the following modifications: the 25μL endpoint PCR reaction was comprised of 12.5 μL Accustart II PCR Supermix (Cat# 95137-500, VWR, Mississauga, ON, Canada); 0.5 μL of each forward and reverse primer was used (final concentration 0.2 μM per primer, 2.0 μL final volume); and 75 ng of DNA and nuclease-free water was included. We confirmed *L. passim* infections occurred only in treatments that were inoculated with *L. passim* using quantitative (q) PCR to detect copies of the *L. passim* cytochrome b gene in each NEB. The qPCR reactions consisted of SSoAdvanced™ Universal SYBR® Green Supermix (BioRad Laboratories, Hercules, USA), genomic DNA, nuclease-free water, and LpCytb_F2, and LpCytb_R primers (Vejnovic et al. 2018) with RpS5 as a reference

gene (Gisder and Genersch 2013). Amplification assays were performed in triplicate in a CFX384 Touch™ Real-Time Detection System (BioRad Laboratories, Hercules, USA). Thermal cycler settings were 3 min at 98°C for initial denaturation/enzyme activation followed by 40 cycles of 10 sec at 98°C and 20 sec at 60°C. Specificity was checked by performing a melt-curve analysis from 65-95°C in increments of 0.5°C at 2 sec per step.

2.2.5 Sucrose responsiveness assay

Individual NEBs were orally-inoculated as above (see 2.2.3 Survival). NEBs that did not readily consume their inoculum were discarded, while those that did were caged according to treatment. Each treatment consisted of one cage per replicate per year (total of 4 replicates per treatment). In 2019, cages consisted of 30 bees per treatment, while in 2020 and 2021, cages consisted of 40 bees per treatment. Cages were maintained and provisioned as above, with dead bees being removed from cages as they appeared. At 16 days post-inoculation (dpi), the inoculated NEBs were prepared for the sucrose responsiveness assay.

At 16 dpi inoculated NEBs were starved in their cages for 60 min prior to being collected and briefly cold anesthetized on ice until immobile (Mustard et al. 2012). Each inoculated NEB was then restrained in a harness (a cut-off portion of a drinking straw) using a thin piece of parafilm placed between the head and the thorax (see Supplementary Figure 1 and Scheiner et al. 2013 for an additional example). Special care was taken to ensure that the inoculated NEBs could still freely move their proboscises and antennae after restraint. These restrained NEBs were then starved for an additional 4.5-5 hrs before the sucrose responsiveness assay began. The antennae of restrained NEBs were presented with a concentration series of 0.1, 0.3, 1, 3, 10, and 30 % sucrose (Page et al. 1998) with 60% as a positive control. NEBs were assayed in ascending order of sucrose concentration to decrease potential sensitization that can occur with

higher sucrose concentrations. After each sucrose presentation, water was provided to the antennae to control for sensitization or habituation (Page et al. 1998; Mustard et al. 2012). The interstimulus interval (interval between successive sucrose concentrations) varied between 1-2 min depending on the number of individuals being assayed at any one time, usually between 10-25. A NEB was observed to 'respond' by fully extending its proboscis when a drop of sucrose was touched to its antennae. Small movements of a proboscis that did not result in full extension were not considered responsive. NEBs that responded to water, responded inconsistently, or failed to respond to 60% sucrose were excluded from further analyses. After the sucrose responsiveness assay was complete, all NEBs that responded to the assay were frozen at -20°C for further processing.

2.2.6 Confirmation and quantification of infection

We confirmed infection, quantified parasite load, and ensured cross-contamination did not occur in 4 randomly chosen NEBs per treatment that were assayed for sucrose responsiveness in 2019 and 2021. We confirmed *N. ceranae* was the only *Nosema* spp. present, and only present in treatments that contained *N. ceranae*-inoculated bees as above. We also quantified the number of *N. ceranae* spores/mL using a Helber Z3000 counting chamber as above (see 2.2.1 *Nosema ceranae)*, and detected copies of the *L. passim* cytb gene/NEB using the same technique as above (Confirmation of infection-(survival experiment)), but then also quantified the number of copies of the *L. passim* cytb gene/NEB via absolute quantification using the standard curve method. Standard curves were prepared from plasmids harbouring the target amplicons with copy numbers diluted from 10^8 to 10^2 (see Supplementary Table 2.3).

2.2.7 Statistical Analyses

Statistical analyses were performed in 'R'studio v.4.2.1 for Mac OS X (R et al. 2022). Survival curve data were analyzed using a Mixed Effects Cox Model (coxme, 2.2-18.1, coxme) with treatment as the predictor variable, and 'cage' nested with 'replicate' as a random effect. This was then followed by ANOVA (Anova, 3.1-0, car) to determine if there was an effect of treatment on survival. Post-hoc tests were then completed using emmeans (emmeans, 1.8.5, emmeans) with a Benjamini Hochberg correction for multiple comparisons to differentiate between treatment effects. Sucrose responsiveness data were analyzed using a generalized linear mixed effects model with a binomial distribution. Response to sucrose was used as the response variable, parasite treatment as the predictor variable, and 'bee' as a random effect. The significance of the predictor variable was evaluated using an *F*-test (Anova, 3.1-0, car), and multiple comparisons were performed (glht, 1.4-20, multcomp). Model fit was assessed by plotting the scaled residuals, examining Levene's test for homogeneity of variance, and Kolmogorov-Smirnov test for overdispersion (simulateResiduals, 0.4.6, DHARMa). SRS were calculated by summing the number of sucrose concentrations in the series to which a bee responded by extending her proboscis (Pankiw et al. 2004). SRS were then evaluated using a Kruskall-Wallis rank sum test followed by Dunn's test of multiple comparisons (Dunn.test, 1.3.5, dunn.test) to determine if treatment had an effect on SRS. We also compared parasite density between single and mixed infection treatment groups within and between years for bees from the sucrose responsiveness assay using Welch's t-tests.

2.3 Results

2.3.1 Survival

For inoculated newly-emerged bees (NEBs), there was an effect of treatment on survival (χ^2_4=353.2, *P*<0.001, Fig. 2.1). Newly-emerged bees inoculated with a parasitic treatment had significantly shorter lifespans than NEBs inoculated with sucrose or media control treatments. The *N. ceranae*-only treatment had the most negative effect on inoculated NEB survival, followed by the mixed-infection treatment, and then *L. passim*-only treatment. NEBs inoculated with *N. ceranae* experienced >50% survival only four days earlier than NEBs inoculated with the mixed infection, eight days earlier than NEBs inoculated with *L. passim* only, and eight and 11 days earlier than NEBs inoculated with media and sucrose control groups respectively (Fig. 2.1). There was no difference in survival between sucrose and media control-inoculated NEBs (Fig. 2.1).

We confirmed the infection status of 60 NEBs across all 5 treatments and 4 replicates. All NEBs examined from the sucrose and media control treatments were free of both *N. ceranae* and *L. passim* across all replicates. All NEBs examined from the *N. ceranae* only, *L. passim* only, and mixed infection treatments were positive or negative for their respective treatments. No cross-contamination was observed.

2.3.2 Sucrose responsiveness assay

Overall, there was an effect of parasite treatment on honey bee responsiveness to sucrose (χ^2_4=39.686 *P*<0.001, Fig. 2.2) as well as on the SRS of individual bees (χ^2_4=39.556 *P*<0.05, Fig. 2.3). Parasitism in general significantly increased both honey bee responsiveness to sucrose and SRS when compared to control honey bees.

We confirmed the infection status and parasite density of 41 randomly chosen NEBs assayed for sucrose responsiveness at 16 dpi in 2019 and 2021 (4 NEBs per treatment per year, except for the *L. passim*-only treatment in 2021 where 5 NEBs were examined (see Supplementary Table 2.4 for densities). There was no difference in *N. ceranae* spore density between *N. ceranae* only and mixed infection NEBs in 2019 (*t=1.07,* df=5.20*, P*=0.33) or 2021 (*t=0.77,* df=3.20, *P*=0.49), and there was no difference in spore density between years for the *N. ceranae* only treatments (*t= 2.00,* df=4.64, *P*=0.11) or mixed infections (*t=1.09*, df=3.30, *P*=0.35). There was no difference in *L. passim* cytb density between *L. passim* only and mixed infection NEBs in 2019 (*t=0.04,* df=4.41, *P*=0.97) or 2021 (*t=2.09*, df=3.00, *P*=0.13), and there was no difference in *L. passim* cytb density between years for the *L. passim* only treatments (*t=2.56*, df=3, *P*=0.08) or mixed infections (*t=1.14*, df=3.01, *P*=0.34) (see Table 2.1). All NEBs examined from the sucrose and media control treatments were negative for both *N. ceranae* and *L. passim*. All NEBs examined from the *N. ceranae* only, *L. passim* only, and mixed infection treatments were positive or negative for their respective treatments, and no cross-contamination was observed (Supplementary Table 2.4).

2.4 Discussion

This study is the first to examine the effects of locally-obtained single and mixed *N. ceranae* and *L. passim* infections under controlled inoculation conditions on honey bee survival and energetic stress. The survival curve which followed parasite-inoculated and uninoculated NEBs to total mortality showed that honey bee lifespan was negatively affected by both single and mixed parasitic infections. Though we did experience some total mortality of bees in specific cages early in this experiment, this was attributable to clogged sucrose feeders, rather than the effects

of parasitization. Our sucrose responsiveness assay illustrated that inoculation with both single and mixed infections significantly increased honey bee responsiveness to sucrose, regardless of the infection type. This increased responsiveness was driven by the high SRS that parasitized NEBs had compared with control NEBs, which suggests that parasitized NEBs are experiencing higher levels of hunger due to increased energetic stress caused by the presence of parasites. These findings add to the body of literature which indicate *N. ceranae* is virulent in honey bees (especially to inoculated NEBs maintained in cages on liquid carbohydrates only), and the small but growing body of literature that suggests *L. passim* is pathogenic to honey bees on its own, but is not as virulent as *N. ceranae* (Cornman et al. 2012; Ravoet et al. 2013; Jack et al. 2016; Goblirsch 2018; Martín-Hernández et al. 2018; Liu et al. 2019; Arismendi et al. 2020; Gómez-Moracho et al. 2020; Liu et al. 2020; Buendía-Abad et al. 2021).

Currently, the literature is divided as to whether interactions between *N. ceranae* and *L. passim* occur due to their geographic separation within the honey bee digestive tract (Tritschler et al. 2017; Arismendi et al. 2020). Tritschler et al. 2017 hypothesized no interaction occurs between *N. ceranae* and *L. passim* based on parasite quantities in field-collected honey bees, while Arismendi et al. (2016) suggested that synergism occurs between the parasites based on a honey bee survival curve experiment. In the current study, contrary to our prediction, NEBs inoculated with the mixed infection had longer lifespans than NEBs inoculated with *N. ceranae* only, and shorter lifespans than NEBs inoculated with *L. passim* only. This finding supports neither of the previously mentioned hypotheses, but instead supports the concept that immunomodulation is occurring (i.e. stimulation, or in this case, suppression of parts of the immune system), which is what Schwarz and Evans (2013) found in honey bees that had been

inoculated with *N. ceranae* and *C. mellificae*. Although the previous authors did not monitor honey bee survival or parasite density, the patterns observed in their gene expression study mimic the patterns of NEB survival in our survival curve study having similar treatment groups. NEBs inoculated with a mixed infection in Schwarz and Evans (2013) mounted a more moderate response to infection (three antimicrobial peptides [AMPs] induced) than NEBs inoculated with *N. ceranae* only (five AMPs), and a more severe response than NEBs inoculated with *C. mellificae* only (2 AMPs). Though we did not collect gene expression data, the phenomenon of increased survival and more moderate immune responses after inoculation with mixed infections has also been observed in other related host-parasite systems. In *Rhodnius prolixus*, bugs inoculated with both *Trypanosoma cruzi* and *T. rangeli* had increased survival, reproduction, and overall fitness compared to those inoculated with either *T. cruzi* or *T. rangeli* alone (Peterson et al. 2016). *Rhodnius prolixus* inoculated with both *T. cruzi* and *Beauvaria bassiana* exhibited increased survival compared to those inoculated with *T. cruzi* only (Garcia et al. 2016), while *Meccus pallidipennis* inoculated with both *T. cruzi* and *Metarhyzium anisopliae* had increased survival compared to those inoculated with either *T. cruzi* or *M. anisopliae* alone, and lower levels of phenyloxidase in hemolymph compared to those inoculated with only *T. cruzi* (Flores-Villegas et al. 2020). Garcia et al. (2016) and Peterson et al. (2016) suggested that *T. cruzi* exerts a protective effect against fungal infections as well as other trypanosomatid infections. The patterns of NEB survival observed in our survival curve study mimic the patterns of gene expression observed in Schwarz and Evans (2013), and collectively suggest immunomodulation is occurring, and that trypanosomatids may have a protective effect against

N. ceranae. However, future experiments should endeavour to include gene expression data along with survival data to fully support this hypothesis.

Additionally, it is possible that synergism was not observed in our study as it was in Arismendi et al. (2020) due to differences in experimental design. Local *L. passim* and *N. ceranae* strains were used in both studies, meaning differences in results could, in part, be due to strain variation. In our study, we individually inoculated NEBs with *N. ceranae* and *L. passim* to ensure they received the desired density of both parasites (1.0 or 1.2×10^5 respectively). Conversely, Arismendi et al. (2020) used NEBs obtained from colonies naturally infected with *L. passim* at a density of $1.0\pm0.6\times10^3$, and then individually inoculated NEBs with *N. ceranae* at a density of $1.0\pm0.3\times10^5$ as required. The differing densities of *L. passim* used in each experiment, as well as the differences in the order of parasite inoculation could have also contributed to variation in survival (Garcia et al. 2016). Differences in the diets that the NEBs were provisioned could also have influenced NEB survival. Honey bees infected with *N. ceranae* when provisioned on high quality pollen (protein) diets, exhibit increased survival despite an increased spore load, compared to those provisioned on low quality or no pollen diets (Porrini et al. 2011b; Di Pasquale et al. 2013; Jack et al. 2016). The increased quantity and quality of pollen found in our pollen patties could be a factor contributing to the increased survival of NEBs inoculated with both *N. ceranae* and *L. passim* compared to those in Arismendi et al. (2020). One final reason Arismendi et al. (2020) may have observed a synergism that we did not is due to the difference in the length of the two experiments. The duration of the survival curve for Arismendi et al. (2020) was 20 days, whereas the survival curve in the current study ended

with total mortality. It is possible that if Arismendi et al. (2020) increased the length of their experiment, that the results of the two studies would have been more similar.

Though the results of the sucrose responsiveness assay did not completely reflect our prediction, the fact that parasite-inoculated NEBs had increased responsiveness to sucrose and higher SRS than control-inoculated NEBs is not surprising as parasites do possess the ability to modify the behaviour and physiology of their hosts (Thompson and Kavaliers 1994; Poulin 2010). *Nosema ceranae* increases energetic stress (via sucrose responsiveness and molecular markers) and decreases the lifespan of honey bees (Mayack and Naug 2009; Goblirsch 2018), both of which we observed in the current study. *Lotmaria passim* also increases energetic stress (assessed via molecular markers) and may decrease the lifespan of bees (Arismendi et al. 2020; Gómez-Moracho et al. 2020; Liu et al. 2020; Buendía-Abad et al. 2021). Given *that L. passim* appears to be less virulent than *N. ceranae*, it was surprising to see NEBs inoculated with the *L. passim*-only treatment having (numerically) higher SRS than NEBs inoculated with *N. ceranae* only, despite having a longer lifespan. This finding may correspond to *L. passim*-infected bees having higher levels of hunger, and in turn, increased energetic stress. Taken together with the longer lifespan, this suggests that honey bees infected with only *L. passim* may be able to better compensate for the long-term negative effects of infection (e.g., decreased lifespan) simply by consuming greater quantities of resources when they are present, which could be quantified in future experiments. This type of compensation has been observed several times in Hymenoptera under various starvation and infection scenarios (Rinderer 1977; Moret and Schmid-Hempel 2000; Basualdo et al. 2014; Kay et al. 2014; Jack et al. 2016). The (numerically) lower SRS of bees infected with *N. ceranae* either alone or in the mixed infection

indicate that if these bees are compensating for the negative effects of infection via diet consumption, that the quality of resources may also play a role in the bees' ability to compensate when *N. ceranae* is present. The influence of protein (pollen) quality in *N. ceranae* infections has been observed previously (Jack et al. 2016). Bees that were infected with *N. ceranae* that were provisioned on the highest quality protein diet consumed more diet than *N. ceranae*-infected bees provisioned on lower quality diets (Jack et al. 2016). In addition to being a highly virulent parasite, *N. ceranae* is also dependent on its host's nutritional status for development because it is amitochondriate (Goblirsch 2018). Therefore, having a bee receive and provide better quality nutrition should be to the benefit of *N. ceranae* (and the bee), and may be why we observed (numerically) lower SRS in bees infected with *N. ceranae*.

We observed no significant difference in parasite density across treatments or years for honey bees at the end of the sucrose responsiveness assay (16 dpi). This finding, along with the increased lifespan for NEBs inoculated with *L. passim* alone, supports the previously mentioned trade-off, where the presence of *L. passim* seems to allow honey bees to compensate for the negative effects of infection via increased food consumption. Increased sucrose consumption has been both suggested and observed for honey bees infected with other parasites such as *N. ceranae* (Mayack and Naug 2009; Naug and Gibbs 2009). Additionally, honey bees infected with *N. ceranae* with access to high quality pollen diets as adults have increased survival (Di Pasquale et al. 2013; Basualdo et al. 2014; Fleming et al. 2015; Jack et al. 2016) and spore loads compared to uninfected bees (Porrini et al. 2011b; Basualdo et al. 2014; Jack et al. 2016). For bees inoculated with *N. ceranae* either alone or with the mixed infection in our experiment, the fact that no differences in parasite densities across treatments were seen, coupled with

decreased lifespans, again suggest that resource quality may also play a role in the ability of bees to compensate for infection. We did not manipulate diet quality and have neither sucrose nor pollen consumption data for the current experiment. It is reasonable to assume that honey bees with higher SRS (parasitized bees) would also consume more sucrose, and perhaps pollen as young adults, potentially allowing them to immunomodulate and increase survival (Alaux et al. 2010; Di Pasquale et al. 2013). To determine if increased food consumption and/or diet quality leads to infected honey bees having longer lifespans via immunomodulation, one could manipulate diet quality, and monitor consumption as well as immune gene expression and survival over time. Given that *N. ceranae* is an intracellular, amitochondriate parasite that depends on the nutritional status of the honey bee for development (Goblirsch 2018), we would expect to see increased consumption of higher quality diet (sucrose and pollen), and better immunomodulation by the bees consuming high quality diet. Because *L. passim* is an extracellular parasite that may use glucose as a source of energy (Runckel et al. 2014), we would expect to see consumption, particularly of sucrose, increase with decreasing quality, and immunomodulation to be stable across diet treatments as long as bees could vary their consumption accordingly.

Initially, we were surprised at the disparity between *N. ceranae* and *L. passim* densities within the NEBs examined from the sucrose responsiveness assay because similar dosages and the same inoculation technique were used. However, given that *N. ceranae* is an intracellular parasite of the honey bee midgut, and *L. passim* is an extracellular parasite of the honey bee hindgut, differences in density could be related to differences in reproductive strategies, and the length of time required for the parasites to complete a reproductive cycle. In a lepidopteran cell

line, *N. ceranae* is able to complete its life cycle in 96 hours (Gisder et al. 2011). Though we do not know the length of time *L. passim* requires to complete its life cycle within the honey bee digestive tract, or in culture media, we do know that *L. passim* cell densities in culture media can range from more than 20× less to 33× more than the initial inoculum density at 96 hours after inoculation depending on the culture media used (Gómez-Moracho et al. 2020). Furthermore, though both parasites could be transmitted via a fecal-oral route (Schwarz et al. 2015; Goblirsch 2018; Buendía-Abad et al. 2021) it is much more likely that a *L. passim* infection could be lost or reduced via a defecation event compared to a *N. ceranae* infection, due to its presence in the hindgut, leading to lower parasite density.

This study has illustrated that under standardized cage conditions, single and mixed *N. ceranae* and *L. passim* infections negatively affect honey bee survival, and their responsiveness to sucrose. These results confirm that *N. ceranae* is a highly virulent honey bee parasite (Higes et al. 2007; Antúnez et al. 2009; Mayack and Naug 2009; Goblirsch et al. 2013; Schwarz and Evans 2013; Gage et al. 2018; Goblirsch 2018), and support what is currently known about *L. passim*, which is that the parasite is pathogenic to honey bees, but less virulent than *N. ceranae* (Arismendi et al. 2020; Liu et al. 2020; Buendía-Abad et al. 2021). Further studies are required to determine if the negative effects of the parasites observed in this study remain under differing experimental conditions such as parasite inoculation order, cell culture passage number, and parasite strain variation. Buendía-Abad et al. 2021 found that long-term *in vitro L. passim* cultures obtained from culture collections had reduced virulence compared to locally-obtained strains, because of increased cell culture passages. The benefits of using parasite strains obtained locally are twofold: 1) cell culture passage numbers are known and 2) virulence

in local host populations can be determined. Additionally, further work should determine if the negative effects of the parasites observed at the cage and individual-level in this study translate to the colony-level, which would warrant the development of novel parasite management strategies. Because honey bee colonies have a strong buffering capacity (Straub et al. 2015), it is possible that the effects observed in the current study may not translate into the field. However, it is also possible that the cage-level effects would translate, as we have recently seen with *N. ceranae* and its effect on honey bee mortality (Goblirsch et al. 2013; Punko et al. 2021). Decreased lifespans, and increased responsiveness to sucrose could manifest as precocious foraging, and smaller, less-productive populations at the colony-level, which we have seen before with *N. ceranae* (Goblirsch 2018). If precocious foraging and smaller populations are observed, particularly for colonies infected with *L. passim* or *L. passim* and *N. ceranae*, novel management strategies, such as those involving the application of phytochemicals should be further explored (Palmer-Young et al. 2022).

Our study, for the first time, illustrates the negative effects of single *L. passim* and mixed *L. passim* and *N. ceranae* infections on honey bee survival and sucrose responsiveness under controlled inoculation conditions with local parasite strains. Based on the results of this study, we recommend that beekeepers continue to monitor their colonies for *N. ceranae*, and begin to routinely monitor for *L. passim* in an effort to improve honey bee health by correlating parasite diagnosis with colony-level changes that could affect survival and productivity.

2.5 Figures and Tables

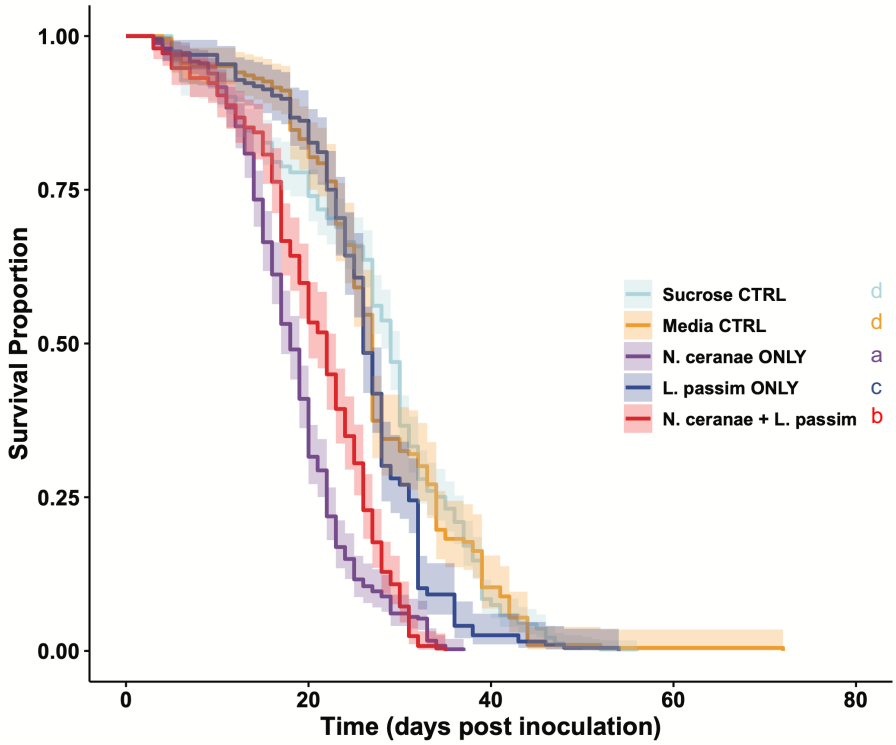


Figure 2.1 Survival of NEBs inoculated with one of five treatments modeled using a Mixed Effects Cox Model. Dark lines represent mean treatment survival across replicates, while the shading surrounding the dark lines represent 95% C.I. Different letters represent significant differences among treatments (coxme; α=0.05)

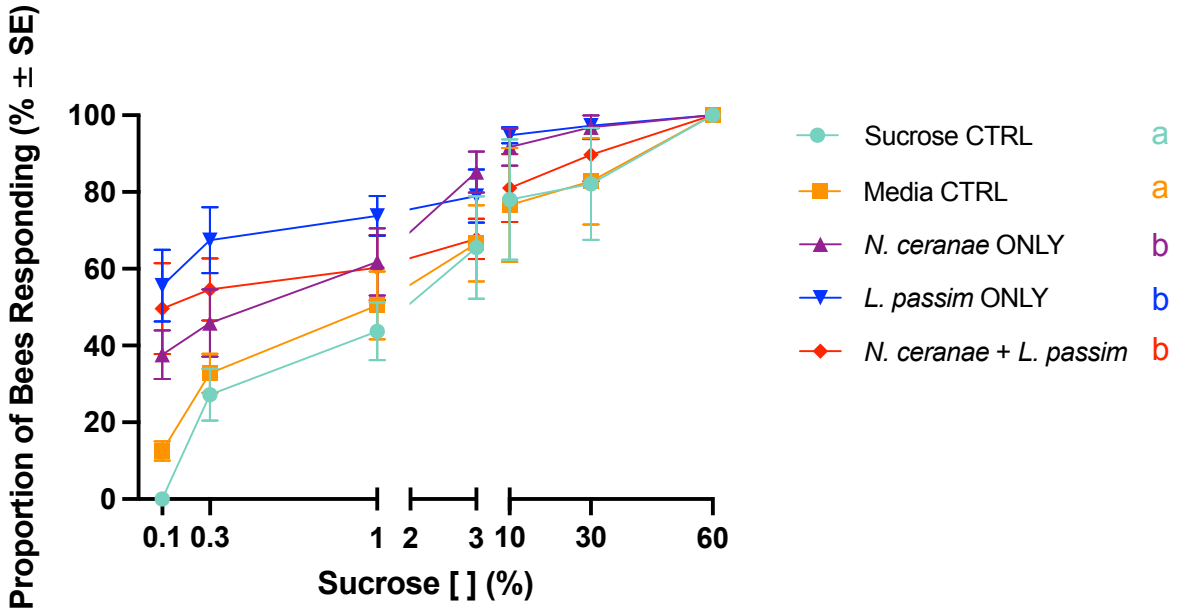


Figure 2.2 Proportion of NEBs inoculated with one of five treatments responding to a sucrose gradient at 16 dpi. Each point represents the mean treatment response across four replicates ± SE, with different letters representing significant differences among treatments (glmer; α=0.05)

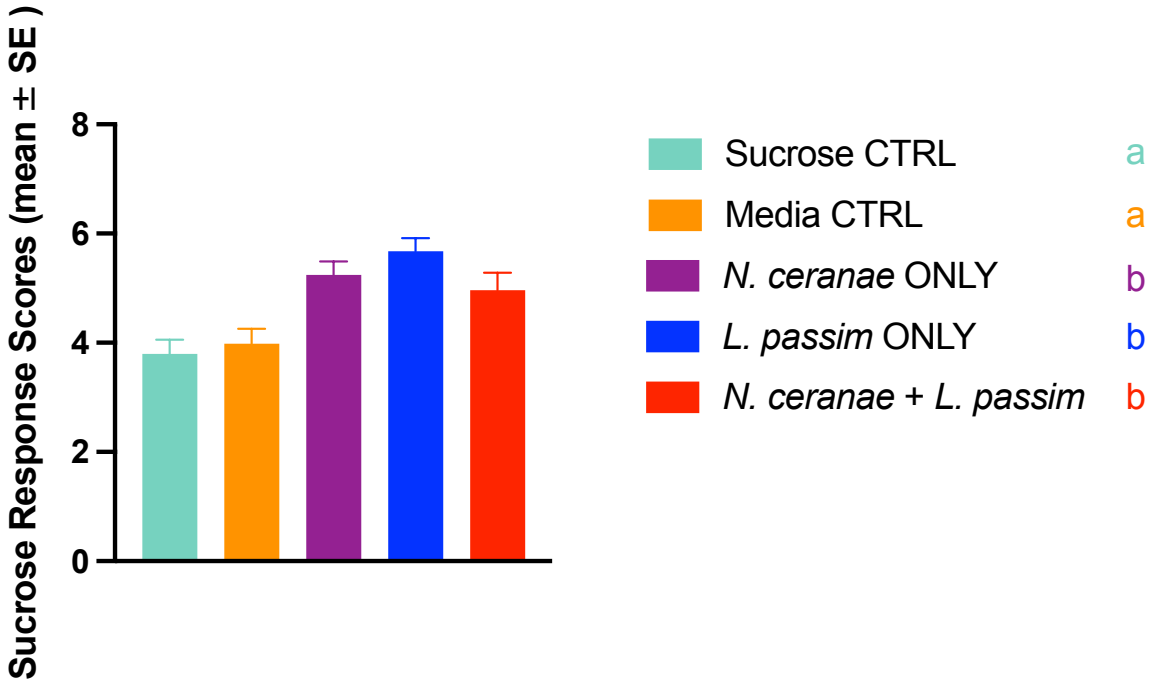


Figure 2.3 Sucrose responsiveness scores (SRS) for bees inoculated with one of five parasite treatments at 16 dpi. SRS were calculated by summing the number of sucrose concentrations in the series to which a bee responded by extending her proboscis. Each bar represents the mean treatment score across four replicates ± SE, with different letters representing significant differences among treatments (dunn.test; α=0.05)

Table 2.1 Parasite densities for honey bees examined at the end of the sucrose responsiveness assay (16dpi). After the sucrose responsiveness assay was complete, all responding NEBs were individually frozen at -20°C until processing occurred. At processing, 4 (or 5) NEBs were randomly chosen from each treatment and year to confirm infection status and density via microscopy (*N. ceranae*, spores/bee) and qPCR (*L. passim*, cytb copies/bee). No significant differences in parasite density exist among treatments or across years

Year	Parasite	Treatment	Mean density ± SE	n
2019	*N. ceranae*	*N. c*	$7.05\times10^7 \pm 8.43\times10^6$	4
2019	*N. ceranae*	*N. c* + *L. p*	$4.49\times10^7 \pm 5.56\times10^6$	4
2019	*L. passim*	*L. p*	$2.12\times10^6 \pm 8.29\times10^5$	4
2019	*L. passim*	*N. c* + *L. p*	$2.74\times10^6 \pm 2.14\times10^6$	4
2021	*N. ceranae*	*N. c*	$8.98\times10^7 \pm 4.60\times10^6$	4
2021	*N. ceranae*	*N. c* + *L. p*	$1.10\times10^8 \pm 2.50\times10^7$	4
2021	*L. passim*	*L. p*	$1.87\times10^3 \pm 5.69\times10^2$	5
2021	*L. passim*	*N. c* + *L. p*	$1.57\times10^5 \pm 7.44\times10^4$	4

Chapter 3 Effects of *Nosema ceranae* and *Lotmaria passim* on antimicrobial peptide expression in honey bees

3.1 Introduction

The Western honey bee (*Apis mellifera* L.) is one of the world's most intensively managed pollinators, required for the pollination of a variety of agricultural crops. Unfortunately, this pollinator faces a diverse pathosphere, which includes the parasites *Nosema* (*Vairimorpha) ceranae* and *Lotmaria passim* that are commonly associated with negative effects on bee health (Evans and Schwarz 2011). While there are a number of studies detailing the effects of *N. ceranae* infections on the honey bee immune response (Antúnez et al. 2009; Chaimanee et al. 2012; Schwarz and Evans 2013; Li et al. 2018), there are few that examine the effects of *L. passim* on the immune response (Arismendi et al. 2020). Both *N. ceranae* and *L. passim* are commonly encountered digestive tract parasites of the honey bee (Higes et al. 2006; Runckel et al. 2011; Williams et al. 2011; Morimoto et al. 2013; Ravoet et al. 2013; Schwarz et al. 2015; Holt and Grozinger 2016; Tritschler et al. 2017). *Nosema ceranae* is a microsporidian parasite that reproduces within the honey bee midgut that was first described in 1996 (Fries et al. 1996), and is now considered to be the dominant *Nosema* spp. in regions where both *N. apis* and *N. ceranae* are present (Chauzat et al. 2007; Paxton et al. 2007; Williams et al. 2008b; Invernizzi et al. 2009; Currie et al. 2010; Stevanovic et al. 2011; Morimoto et al. 2013; Emsen et al. 2016; Punko et al. 2021). *Lotmaria passim* is a recently described trypanosomatid parasite with a preference for honey bee hindgut tissue that was described in 2015 (Schwarz et al. 2015), and also outpaces its relative *Crithidia mellificae* in terms of prevalence (Runckel et al. 2011; Ravoet et al. 2013; Schwarz et al. 2015; Arismendi et al. 2016; Tritschler et al. 2017; Bartolomé et al.

2020). Despite the cosmopolitan distribution of both *N. ceranae* and *L. passim*, we still lack a comparative understanding of how these parasites affect the honey bee immune response, even at the individual level under controlled inoculation conditions, which is disconcerting given that bees can be co-infected with both parasites (Tritschler et al. 2017; Williams et al. 2021). We also do not fully understand how these two parasites interact with one another under controlled inoculation conditions given that they purportedly have different host tissue preferences (Schwarz et al. 2015).

Within individual honey bees, *N. ceranae* is able to degenerate midgut tissues (Dussaubat et al. 2012; Panek et al. 2018), and reduce honey bee lifespan (Higes et al. 2007; Goblirsch et al. 2013; Arismendi et al. 2020; MacInnis et al. 2023). The parasite is also capable of altering honey bee immune responses, foraging behaviour, as well as learning and memory (Antúnez et al. 2009; Chaimanee et al. 2012; Goblirsch et al. 2013; Schwarz and Evans 2013; Huang et al. 2016; Gage et al. 2018; Li et al. 2018). At the colony level, the effects of *N. ceranae* are more obscure, and seem to vary with geographic location. In Germany, studies have shown no correlation between colony mortality and *N. ceranae* infection prevalence (Genersch et al. 2010; Gisder et al. 2010). In the United States metagenomic analyses showed that infection with *N. ceranae* or *N. apis* was a differentiating factor between healthy and colony collapse disorder (CCD) colonies (Cox-Foster et al. 2007; vanEngelsdorp et al. 2009) while *N. ceranae* was only numerically more prevalent in colonies exhibiting CCD (vanEngelsdorp et al. 2009). In Canada, increased *N. ceranae* spore abundance in spring has been correlated with increased winter colony mortality (Punko et al. 2021). In Spain, *N. ceranae* can be present in colonies without causing symptoms (Fernández et al. 2012), but has also been associated with decreased

brood rearing, honey production, colony size, and colony collapse (Higes et al. 2008; Higes et al. 2009; Botías et al. 2013).

Relative to *N. ceranae*, the effects of *L. passim* on individual honey bees are poorly understood, likely due to the organism's recent reclassification (Schwarz et al. 2015). Several studies quantifying the effect of *L. passim* on honey bee lifespan report conflicting findings; some report that the parasite negatively affects lifespan (Liu et al. 2019; Gómez-Moracho et al. 2020; Liu et al. 2020; Buendía-Abad et al. 2021; MacInnis et al. 2023) while others report no effect (Arismendi et al. 2020). Additionally, one study has reported on the effects of *L. passim* on honey bee behaviour, showing that *L. passim*-infected honey bees have increased responsiveness to sucrose compared to uninfected controls (MacInnis et al. 2023). At the colony level, *L. passim* has been found concurrently with *N. ceranae* (Runckel et al. 2011; Ravoet et al. 2013; Tritschler et al. 2017; Williams et al. 2021). While single *L. passim* infections have been correlated with increased winter colony mortality (Ravoet et al. 2013), little is known regarding the effects of single *L. passim* or mixed *L. passim* + *N. ceranae* infections at the colony level. What is known is not encouraging, as recently these infections have been associated with decreased vitellogenin levels, and mixed infections reduce average foraging age at the colony level (MacInnis 2024).

Individual honey bees possess diverse mechanisms to defend themselves against attack from parasites such as *N. ceranae* and *L. passim* (Evans et al. 2006). One of these mechanisms is the humoral defense response, which is part of the innate immune system (Evans 2006; Evans et al. 2006; Antúnez et al. 2009) The humoral defense response encompasses the synthesis and release of antimicrobial peptides (AMPs) from the fat body, or locally through hemocytes and

gut epithelial cells (Bulet and Stöcklin 2005). There are at least five well-characterized AMPs that have been identified in honey bees after induction by parasitic infections have been induced: apidaecin, abaecin, defensin-1, defensin-2, and hymenoptaecin (Evans et al. 2006). Currently, there are no studies detailing comparative localized humoral defense responses of honey bees infected with *N. ceranae*. *L. passim*, or both under controlled inoculation conditions. This is despite the cosmopolitan distribution, emergent nature, distinct host tissue preferences and localized responses among honey bees infected with *N. ceranae* and *C. mellificae* (Schwarz and Evans 2013). Here, the aim is to describe the localized humoral defense responses of honey bees infected with *N. ceranae*, *L. passim*, or both, by quantifying three AMPs in midgut and hindgut tissues over time. In addition, temporal parasite density is monitored in these tissues in an attempt to determine if the two parasites, particularly *L. passim*, have distinct tissue preferences.

3.2 Methods and Materials

3.2.1 Parasites

An axenic culture of *L. passim* was isolated from the dissected ileum of an adult honey bee worker at the Agriculture and Agri-Food Canada's (AAFC) Beaverlodge Research Farm (55°11'43.0"N; 119 °17'57.3"W). The culture was established in the fall of 2016 and subsequently grown and maintained for use as described in MacInnis et al. (2023). Prior to inoculation, *L. passim* cultures were centrifuged at 200 × *g* for 10 min. After this initial centrifugation step, the supernatant was removed, and filtered through a 0.22 μm filter, while the pellet was resuspended in 1 mL of 1× phosphate-buffered saline (PBS). The filtered supernatant was then centrifuged at 200 × *g* for 10 min, the resulting supernatant was

removed, and any remaining pellet resuspended to 1mL with a 1:10 sucrose (50% *w/v*): PBS (1×) solution. The resuspended *L. passim* pellet was successively washed and centrifuged twice in 1 mL of 1× PBS at 200 × *g* for 10 min. After a final resuspension of the *L. passim* pellet in 1mL of 1× PBS, a count was performed using a Helber Z30000 counting chamber (Cat# Z30000, Hawksley, West Sussex, UK) to estimate the number of motile, flagellated *L. passim* cells/mL of culture.

Nosema ceranae spores were obtained from the dissected midguts of *N. ceranae*-infected adult *A. mellifera* workers at AAFC's Beaverlodge Research Farm; the procedure for spore collection was adapted from MacInnis et al. (2020). After dissection, midguts were manually macerated in 1 mL of 1× PBS in a Stomacher® 80 Biomaster Standard Bag (Cat# BA6040, Seward, West Sussex, UK) for 1 min before maceration in a Stomacher® 80 blender (Cat # 030010019, Seward, West Sussex, UK) for 5 min. This macerate was then passed through a 40μm cell strainer (Cat# 352340, Fisher Scientific) and rinsed with 15 mL of 1× PBS. The resulting filtrate was subsequently vacuum-filtered through a 10 μm separator (Cat# 60344, Pall Corporation, Ann Arbor, MI, USA) and rinsed with another 15 mL of 1× PBS. The resulting 30 mL filtrate was then centrifuged at 800 × *g* for 10 min, and the pellet resuspended in 1 mL of 1× PBS. The 1 mL of *N. ceranae* spores in 1× PBS was then treated with 100 IU/mL Penicillin-100 μg/mL Streptomycin (Cat# 15140122, Fisher Scientific) for 1 hr to kill any contaminating bacteria (Schwarz and Evans 2013; MacInnis et al. 2023). The *N. ceranae* spores were then washed 3 times in 1 mL of 1× PBS followed by centrifugation at 800 × *g* for 10 min. After the final resuspension in 1 mL of 1× PBS, a count was performed using a Helber Z30000 counting chamber to estimate the number of spores/mL (Cantwell 1970). *Nosema* spp. were verified via conventional polymerase chain

reaction (PCR) techniques outlined in van den Heever et al. (2015b) with the following modifications: 200 μL of macerate was used for DNA extractions; 75 ng of total DNA was amplified via PCR; and primers NoscRNAPol-F2/NoscRNAPol-R2 NosaRNAPol-F2/NosaRNAPol-R2 (Supplemental Table 3.1). Thermal cycler settings used in Gisder and Genersch (2013) were used to differentiate between *N. apis* and *N. ceranae*.

3.2.2 Experimental bees

Frames of eclosing worker bees were collected from non-experimental honey bee colonies managed by the Apiculture Program at AAFC Beaverlodge. Between four to six brood frames, from four to six different colonies previously confirmed (via PCR) to be free of *Nosema* spp. and trypanosomatid spp. were maintained in an incubator at 33°C ± 1.0°C (models I36NLC8, I36NLC9, Percival Scientific, Perry, IA). Bees were collected from these frames daily, so that all newly-emerged bees (NEBs) used in the experiment were <24 hrs old, and free of any *Nosema* spp. and trypanosomatid spp. infections.

3.2.3 Inoculation and caging

Individual NEBs were orally inoculated with 5 μL of one of the five following treatment groups diluted in 1:10 sucrose (50% *w/v*): PBS (1×) solution via a 10 μL pipette: sucrose control (1:10 sucrose:PBS solution only), media control (any resulting pellet from the centrifuged *L. passim* supernatant), *N. ceranae* only (1.0×10^5 *N. ceranae* spores), *L. passim* only (1.2×10^5 motile, flagellated *L. passim* cells), and *N. ceranae* + *L. passim* (1.0×10^5 *N. ceranae* spores + 1.2×10^5 motile, flagellated *L. passim* cells). After inoculation, these NEBs were maintained in 15 mL centrifuge tubes for 30 min prior to caging to ensure they consumed the inoculum. After the 30 min had elapsed, NEBs that had not fully consumed their inoculum were discarded, while the remainder were caged by treatment. Each treatment consisted of two cages (A and B) each

containing 35 inoculated NEBs and 35 uninoculated NEBs (thoraxes paint-marked to identify them), the latter to provide social interaction and to act as controls that received minimal handling as in MacInnis et al. (2023). Cages were maintained at 33°C ± 1.0°C in programmable incubators, and NEBs were provisioned with 50% *(w/v)* sucrose from a gravity feeder along with a 25% pollen patty *ad lib*, as per MacInnis et al. (2023), with diet replaced every 72 hours.

3.2.4 Bee collection and tissue sampling

Four inoculated bees were randomly removed from each cage to be processed for gene expression at each of five time points: 1, 2, 3, 6, and 17 days post inoculation (dpi). These bees were placed in centrifuge tubes after collection and briefly anaesthetized on ice. Digestive tracts were then removed using sterile dissection tools, and the tract cut in two places with sterile scalpels: anterior to the midgut (to remove the crop), and posterior to the midgut. This produced two sections, a midgut section and hindgut section (ileum and rectum) (see Supplemental Figure 3.1). Each section was placed into its own sterile 1.5mL microcentrifuge tube on dry ice, and then stored at -80°C until further processing occurred. All bees infected with *N. ceranae* only had died by 17 dpi, so no processing or downstream analyses occurred for this treatment group at this time point.

3.2.5 RNA extraction and cDNA synthesis

Total RNA was extracted from individual midguts using TRIzol and Phasemaker tubes (Cat # 15596018, A33248, Invitrogen, ThermoFisher Scientific, Ottawa, ON, Canada) following the manufacturer's protocol after homogenization with sterile pestles. Total RNA was extracted from individual hindguts using the Qiagen Rneasy ® Lipid Tissue Mini Kit (Cat # 74804, Qiagen, Valencia, CA, USA) following the manufacturer's protocol after homogenization with sterile pestles. Following extraction, purified RNA pellets were resuspended in nuclease-free water,

and quality and quantity checked using a Nanoquant 200 (Tecan Infinite®, Morrisville, NC, USA). After quantification, 400ng of total RNA were aliquoted for DNA degradation using DNAse I (RNAse-free) (Cat# AM2224, Ambion, Life Technologies, ThermoFisher Scientific, Ottawa, ON, Canada), followed by first strand cDNA synthesis using SuperScript II Reverse Transcriptase and RNaseOUT (Cat # 18064071, 10777019, Invitrogen, ThermoFisher Scientific, ON, Canada) following the manufacturer's protocol. Random hexamers (100ng) and oligo $(dT)_{12\text{-}18}$ (50ng) (Cat # N8080127, 18418012, Invitrogen, ThermoFisher Scientific, Ottawa, ON, Canada) were used to prime transcription. After transcription was complete, cDNA was diluted 1:5 in nuclease-free water.

3.2.6 RT-qPCR for absolute quantification of gene expression

Primers used in this study can be found in Supplementary Table 3.1, and include pairs targeting RpS5, Hymenoptaecin, Apidaecin, Defensin 1, *N. ceranae* PTP3, and *L. passim* LSU. Data were normalized to RpS5 as it proved to be stable across all treatments and timepoints. qRT-PCR analyses were performed in 384-well clear/white plates with Microseal 'B' adhesive seals using the Bio-Rad CFX384 real time system (Cat # HSP3805, MSB1001, Bio-Rad, Mississauga, ON, Canada). Each plate was comprised of 112-120 cDNA samples assessed for one target. Interplate controls were run on each plate to monitor for any between-run variation. No template control (NTC) reactions were run on each plate to monitor contamination, and positive controls for each target were included on each plate assessing the target using serial dilutions (2.00×10^2-2.00×10^7 copies) of sequence-verified recombinant clones (Supplementary Table 3.2) to monitor amplification efficiency, primer efficiency, and to generate standard curves for each target (Supplementary Table 3.1). All samples were run in duplicate or triplicate, and the protocol and analysis for this portion of the study were based on recommended guidelines

(Bustin et al. 2009). All reactions contained equal amounts of template cDNA (2μL of 1:5 diluted cDNA, 1:100 diluted cDNA for samples requiring further dilution, or nuclease-free water for NTCs), 200nM each of a forward and reverse primer, and 1× SsoAdvanced Universal SYBR® Green Supermix (Cat # 1725274, Bio-Rad, Mississauga, ON, Canada). Thermalcycler conditions were as follows: 97°C for 1 min, followed by 40 cycles of 95°C for 2 sec, 60°C for 5 sec, and melt curve analysis from 65-95°C at 0.5°C / 5 sec increments to confirm expected dissociation curves. All RT-qPCR experiments and analysis were handled by the same individual to minimize any potential handler variation as in MacInnis (2024).

The average copy number / bee for each target gene for each tissue type, at each time point, for each bee was calculated, then the average copy number of the target gene / bee was normalized to its corresponding average RpS5 value for each bee, and then normalized to the average number of copies of RpS5 across all bees for the tissue type and time point being analyzed:

$$\left(\frac{Average\ target\ copies/bee}{Average\ RpS5\ copies/bee}\right) * (Average\ copies\ of\ RpS5/all\ bees^{\dagger})$$

†= Average copies of RpS5 / all bees per tissue type and time point being analyzed.

3.2.7 Statistical analyses

All statistical analyses were performed in 'R' v. 4.2.1 "Funny Looking Kid" within 'R'Studio v. 2022.07.2 +576 "Spotted Wakerobin" for Mac OS X (R et al. 2022).

3.2.7.1 Immune gene expression

To determine if there were tissue-specific effects of parasite treatment on the expression of the three immune genes of interest (apidaecin, hymenoptaecin, and defensin-1), the absolute quantities of each gene and tissue type combination at each of the five time points by parasite

treatment for each gene were compared. Samples that were below the limit of detection or returned a N/A were assigned a value of zero for this analysis. Data were analyzed using linear mixed effects models (lme4, 1.1-31) with treatment as a fixed effect and 'bees' nested within 'cage' as a random effect. Model fit was assessed by plotting the scaled residuals, examining Levene's test for homogeneity of variance, and examining the Kolmogorov-Smirnov's test and Shapiro-Wilk's test for overdispersion (simulateResiduals, 0.4.6, DHARMa and Shapiro.test(resid(), R Core Team 2022). In order to achieve adequate model fit, Box-Cox transformations (including λ=0.5, squareroot transform) (boxcox, 7.3-57, MASS) were applied as necessary prior to models being run. The significance of the fixed effect was evaluated using an *F*-test (Anova, 3.1-0, car), and multiple comparisons were performed (glht, 1.4-20, multcomp). In some of the models generated, random effects were very small (or zero), so the random effect of replicate was removed, and a one-way ANOVA (aov) performed followed by a post-hoc test for multiple comparisons of means when necessary, as above. Datasets that could not be analyzed using linear mixed effects models or one-way ANOVA due to the presence of zeroes were analyzed using a Kruskal-Wallis test followed by Dunn's test of multiple comparisons (dunnTest, 0.9.5, FSA).

3.2.7.2 Quantification of parasites via gene expression

Density of *N. ceranae* infections were compared between *N. ceranae* only infections and *N. ceranae* + *L. passim* infection at each of the five time points per tissue type using a two sample t-test (t.test) or Wilcoxin rank sum test (wilcox.test) where appropriate. Density of *L. passim* infections were compared between the *L. passim* only infection, and *N. ceranae* + *L. passim* infection at each of the five time for the midgut tissue using the Wilcoxin rank sum test as above. Density of *L. passim* infections were compared between the media control group, *L.*

passim only infection, and *N. ceranae* + *L. passim* infection at each of the five time for the hindgut tissue using a one-way ANOVA (aov) or Kruskal-Wallis test as appropriate. The media control was not included in the *L. passim* density comparison for the midgut because samples were below the limit of quantification. Additionally, of the 192 bees collected for gene expression analysis, 11 had low-level parasite contamination in at least one section of the digestive tract and consequently were removed from all gene expression analyses (parasite and immune).

3.3 Results

3.3.1 Immune gene expression

There was only an effect of treatment on apidaecin expression at two of the five measured time points. One at 3 dpi in the midgut, where apidaecin expression was significantly increased in the *N. ceranae* group compared to the media control group (χ^2_4=11.25, *P*=0.024) (Figure 3.1a, Supplementary Table 3.3), and one at 2 dpi in the hindgut where apidaecin expression increased with marginal significance in the sucrose control group compared to media control, *N. ceranae* only, and *N. ceranae* + *L. passim* groups ($F_{4,29}$=2.72, *P*=0.049) (Figure 3.2a, Supplementary Table 3.3).

Hymenoptaecin was differentially expressed in midgut samples only, at 1 and 6 dpi. It was significantly increased in the *N. ceranae* only group compared to the *N. ceranae* + *L. passim* group (χ^2_4=10.58, *P*=0.032) at 1 dpi, and in the *N. ceranae* + *L. passim* group compared to the media control group (χ^2_4=12.83, *P*=0.012) at 6dpi (Figure 3.1b, and Supplementary Table 3.3).

Defensin-1 was only differentially expressed at 1 dpi in both midgut and hindgut tissues. Within the midgut, the *N. ceranae* only group had significantly increased defensin-1 expression

compared to the *N. ceranae* + *L. passim* group (χ^2_4=10.42, *P*=0.033) (Figure 3.1c, Supplementary Table 3.3). In the hindgut, defensin-1 expression was significantly increased in the *N. ceranae* + *L. passim* group compared to the *L. passim* only group at 1 dpi ($F_{4,29}$=3.22, *P*=0.026) (Figure 3.2c, Supplementary Table 3.3).

3.3.2 Confirmation and quantification of parasitic infection

Nosema ceranae PTP3 expression was only differentially expressed between the two treatments where *N. ceranae* was present (*N. ceranae* only and *N. ceranae* + *L. passim*) at 6 dpi in hindgut samples (Figures 3.3 a and b, Supplementary Table 3.3). There was significantly more *N. ceranae* PTP3 expression in the *N. ceranae* + *L. passim* treatment compared to the *N. ceranae* only treatment (W=51, *P*=0.05).

Lotmaria passim LSU was only differentially expressed between the treatments that involved *L. passim* (including the media control). Within midgut tissue, LPLSU was differentially expressed between the *L. passim* only and *N. ceranae* + *L. passim* treatments at 1 and 3 dpi (Figure 3.4a). In both instances, LPLSU expression was significantly increased in the *L. passim* only treatment compared to the *N. ceranae* + *L. passim* treatment (W=48, *P*=0.007, W=52, *P*=0.002 respectively) where it was not quantifiable. Within the hindgut tissue, LPLSU was differentially expressed at all 5 timepoints, where expression was significantly increased in the *L. passim* only and *N. ceranae* + *L. passim* treatments compared to the media control group ($F_{2,20}$=9.95, *P*=0.001, $F_{2,18}$=9.45, *P*=0.002, χ^2_2=12.62, *P*=0.002, $F_{2,21}$=4.43, *P*=0.025, χ^2_2=13.87, *P*=0.001 respectively) (Figure 3.4b, Supplementary Table 3.3).

3.4 Discussion

Nosema ceranae and *L. passim* are two commonly encountered parasites of the honey bee often thought to be geographically separated within the digestive tract (Fries et al. 1996;

Schwarz et al. 2015). This study provides valuable insight regarding the location of these parasites over time under controlled inoculation conditions, along with localized humoral defense responses honey bees employ in response to these parasites. Overall, I found that parasites did not elicit distinct immune responses in honey bees over time in the midgut or hindgut tissues. Though many differences in parasite gene quantities were not observed between treatments in either tissue type over time, it was interesting to see the trends in quantities within treatments over time. In both the *N. ceranae* only and *N. ceranae* + *L. passim* treatments, PTP3 expression increased over time in both midgut and hindgut tissues. For the *L. passim* only and *N. ceranae* + *L. passim* treatments, LpLSU expression decreased over time within the midgut, but remained stagnant overtime in the hindgut, with the exception of the *N. ceranae* + *L. passim* treatment at 3 and 17 dpi, suggesting a hindgut preference for *L. passim*.

The lack of distinct immune responses in the form of AMP expression in bees following parasitic infection, particularly at the earlier time points in this study (1-6 dpi) is consistent with the findings of several other studies which indicate that the parasites, especially *N. ceranae*, are able to evade or suppress the honey bee immune system (Antúnez et al. 2009; Chaimanee et al. 2012; Huang et al. 2016; Li et al. 2018; Arismendi et al. 2020). An increase in AMP expression in infected bees was not observed, particularly for those infected with *N. ceranae*, at the late time point (17 dpi) that others reported (Schwarz and Evans 2013; Li et al. 2018; Arismendi et al. 2020) for several possible reasons. Because all the bees from the *N. ceranae* only treatment had died before the final timepoint (17dpi), differences between this treatment and others could not be evaluated. Additionally, in culture and within bees, *N. ceranae* takes 3-4 days to fill the cytosol of cells, which then rupture releasing new infectious spores (Higes et al. 2007;

Gisder et al. 2011). It is possible that this rupturing event was missed with the late time point, and the production of associated AMPs (Schwarz and Evans 2013). It is also possible that the defense response to *N. ceranae* and *L. passim* is largely systemic rather than localized, and that differences in AMP expression might have been observed at this time point if entire bee abdomens had been evaluated rather than midgut and hindgut tissues. Finally, Schwarz and Evans (2013) evaluated AMP expression in honey bee abdomens as well as midgut and hindgut tissues after infection with both *N. ceranae* and *Crithidia mellificae* (trypanosomatid relative of *L. passim*) at early and late time points after infection. They detected distinct differences in AMP expression by infection type at the abdomen and midgut and hindgut level at early and late time points. Regarding the differences they detected at the midgut and hindgut level, it is possible that similar differences were not detected in this study because of how the two tissues were classified. The midgut section in Schwarz and Evans (2013) was comprised of the midgut and ileum, while in this study it was only the midgut. Their hindgut tissue consisted of only the rectum, while in this study it was comprised of both the ileum and rectum. Additionally, the discrepancies in results could also be attributed to how the data were analyzed. Here, five treatment groups were compared to each other at each of the five time points whereas Schwarz and Evans (2013) had three treatments, where they compared each parasite-treated group to the control at each time point.

Overall, LpLSU expression was lower in midgut tissues compared to hindgut tissues at each time point during the experiment for all treatments involving *L. passim*. This, coupled with the lack of LpLSU expression in the midgut tissues from the *N. ceranae* + *L. passim* treatment group at 1 and 3 dpi support the hypothesis that *L. passim* exhibits a preference for hindgut

tissues (Schwarz et al. 2015; Buendía-Abad et al. 2022). The lack of LpLSU expression in the midgut tissues, and relatively stable levels of LpLSU in the hindgut tissues of the media control treatment indicate that only *L. passim* debris was present within this treatment group, and that replication was not occurring. Additionally, the similar levels of LpLSU expression across tissue types for the *L. passim* only and *N. ceranae* + *L. passim* treatment groups suggest that no resource competition is occurring between these two parasites (Tritschler et al. 2017). This is despite the differences in LpLSU expression in midgut tissues at 1 and 3 dpi where LpLSU was not quantifiable in the *N. ceranae* + *L. passim* treatment group. Interestingly, the similar expression patterns of hymenoptaecin in both the midgut and hindgut tissues for bees in the *L. passim* only and *N. ceranae* + *L. passim* treatments do suggest that the bees could be responding locally to *L. passim* even though we detected few differences between treatment groups at each of the five time points. The increase in hymenoptaecin expression, particularly at the first and last time points could coincide with *L. passim* becoming attached to epithelial cells (Buendía-Abad et al. 2022).

Increasing expression of *N. ceranae* PTP3 in the midgut and hindgut tissues of bees was observed in both the *N. ceranae* only and *N. ceranae* + *L. passim* treatment groups. This indicates rapid reproduction of *N. ceranae* which is commonly observed (Paxton et al. 2007; Forsgren and Fries 2010; Huang and Solter 2013b; Li et al. 2018). No *N. ceranae* PTP3 expression was observed in the midgut or hindgut tissues at 1 dpi for the *N. ceranae* only treatment group, or at 2 dpi in midgut tissues for the *N. ceranae* + *L. passim* treatment group. As PTP3 is involved in the biogenesis of the polar filament during the sporoblast-to-spore stage, and polar filament extrusion (Peuvel et al. 2002), this lack of expression could indicate that PTP3

mRNA was not extracted from spores that were present at these time points, or that PTP3 levels were below the limit of detection for the thermalcycler. The only difference in PTP3 expression between the *N. ceranae* only and *N. ceranae* + *L. passim* treatment groups occurred at 6 dpi in the hindgut tissue, where there was significantly more PTP3 expression in the *N. ceranae* + *L. passim* treatment compared to the *N. ceranae* only treatment group. This pattern has been observed before (MacInnis et al. 2023), and suggests that there may be an interaction occurring when both parasites are present. However, the similar levels of PTP3 expression overall across both treatment groups and tissue types suggest that if an interaction is occurring, it is not a resource competition interaction (Tritschler et al. 2017).

Given the high expression of PTP3 in hindgut tissues, it is possible that *N. ceranae* is reproducing in these tissues, particularly the ileum. The ileum is attached to the midgut via the pylorus, is comprised of six longitudinal folds, a single layer of cuboidal epithelial cells (Santos and Serrão 2006; Kwong and Moran 2016), and is void of the peritrophic matrix which acts as a barrier against *N. ceranae* (de Oliveira et al. 2022). Though *N. ceranae* is thought to be restricted to the midgut for reproduction, studies reporting this often do not include an assessment of hindgut tissues, or quantify spore load or the presence of *N. ceranae* DNA (Fries et al. 1996; Chen et al. 2009; Huang and Solter 2013b). *Nosema ceranae* completes its lifecycle in cell culture (Gisder et al. 2011), can infect honey bee larvae (Eiri et al. 2015), and is cross-infective (Plischuk et al. 2009; Chaimanee et al. 2010; Botías et al. 2012; Fürst et al. 2014; Porrini et al. 2017) indicating that it can complete its lifecycle under varying environmental conditions. To confirm if *N. ceranae* can reproduce within the ileum, further histological studies paying particular attention to the cuticle are required (vanEngelsdorp et al. 2017).

This study illustrates that honey bees do not mount distinct, localized, humoral defense responses with the AMPs hymenoptaecin, apidaecin, and defensin-1 in response to *N. ceranae* and *L. passim* infections under controlled inoculation conditions. This study also shows that there appears to be no resource competition occurring between the two parasites, and further supports the claim that *L. passim* has a preference for the honey bee hindgut over the midgut. The very slightly elevated, but non-significant, LpLSU expression in hindgut tissues at 1, 2, and 6 dpi, and increased PTP3 expression in hindgut tissues at 6 dpi when both *N. ceranae* and *L. passim* are present suggest that there could be an interaction occurring between the two parasites, which would need to be further investigated to confirm.

3.5 Figures and Tables

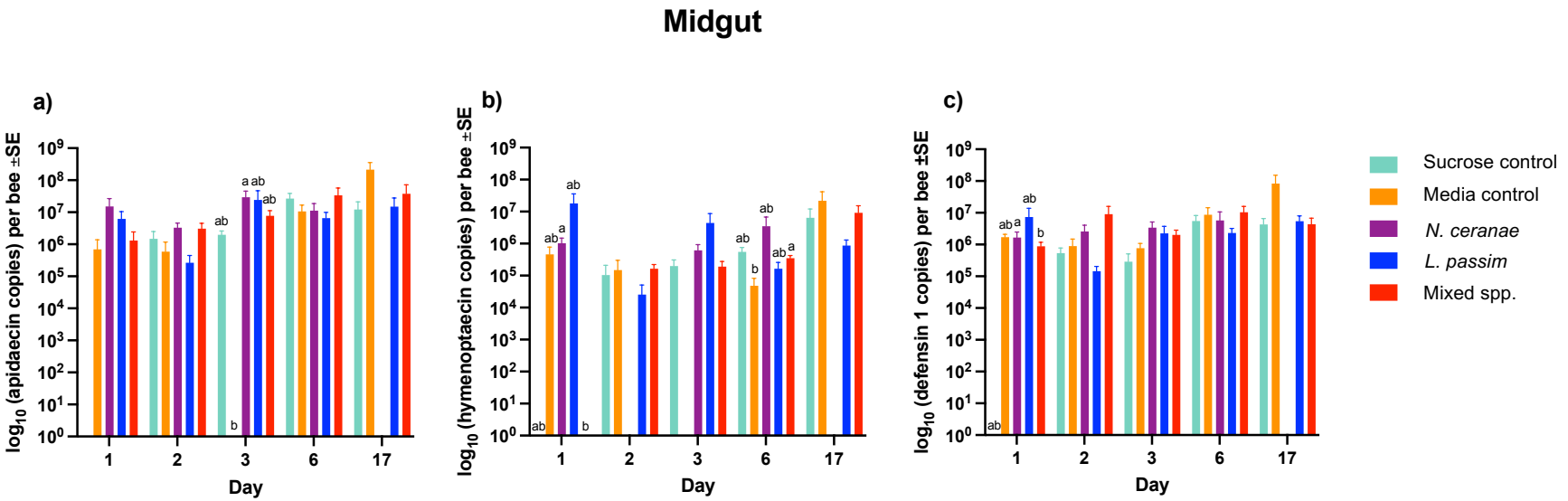


Figure 3.1 Mean (± SE) immune gene expression (copies) in midgut tissues by treatment at 1, 2, 3, 6, and 17 dpi for **a)** apidaecin, **b)** hymenoptaecin, and **c)** defensin-1. No data for *N. ceranae* only at 17 dpi due to all bees dying. Analysis and comparison among treatments performed on raw data. Data were log transformed for visualization. Letters indicate statistically significant differences between treatments (α=0.05). See Supplementary Table 3.3 for statistical comparisons done using linear mixed effects models, one-way ANOVAs, or Kruskal-Wallis tests

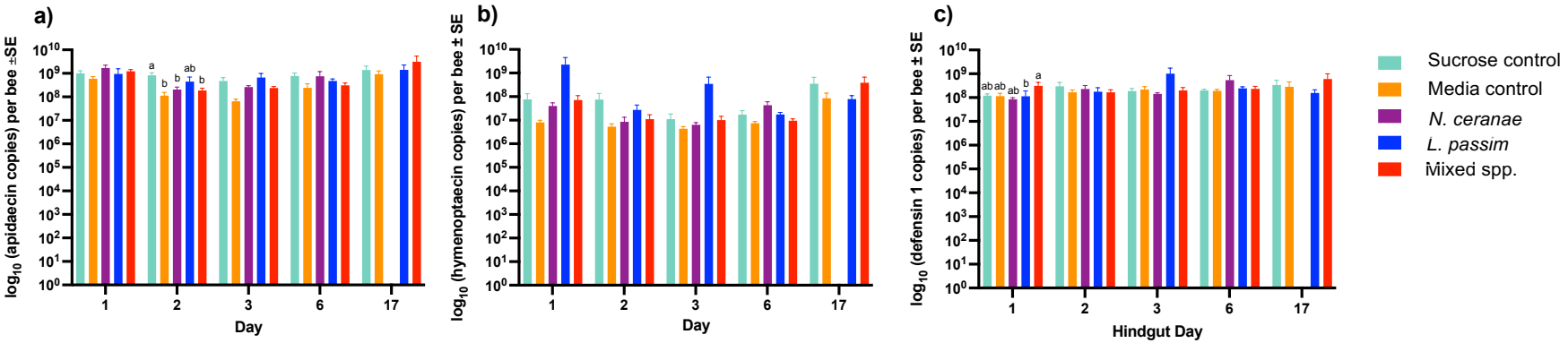


Figure 3.2 Mean (± SE) immune gene expression (copies) in hindgut tissues by treatment at 1, 2, 3, 6, and and 17 dpi for **a)** apidaecin, **b)** hymenoptaecin, and **c)** defensin-1. No data for *N. ceranae* only at 17 dpi due to all bees dying. Analysis and comparison among treatments performed on raw data. Data were log transformed for visualization. Letters indicate statistically significant differences between treatments (α=0.05). See Supplementary Table 3.3 for statistical comparisons done using linear mixed effects models, one-way ANOVAs, or Kruskal-Wallis tests

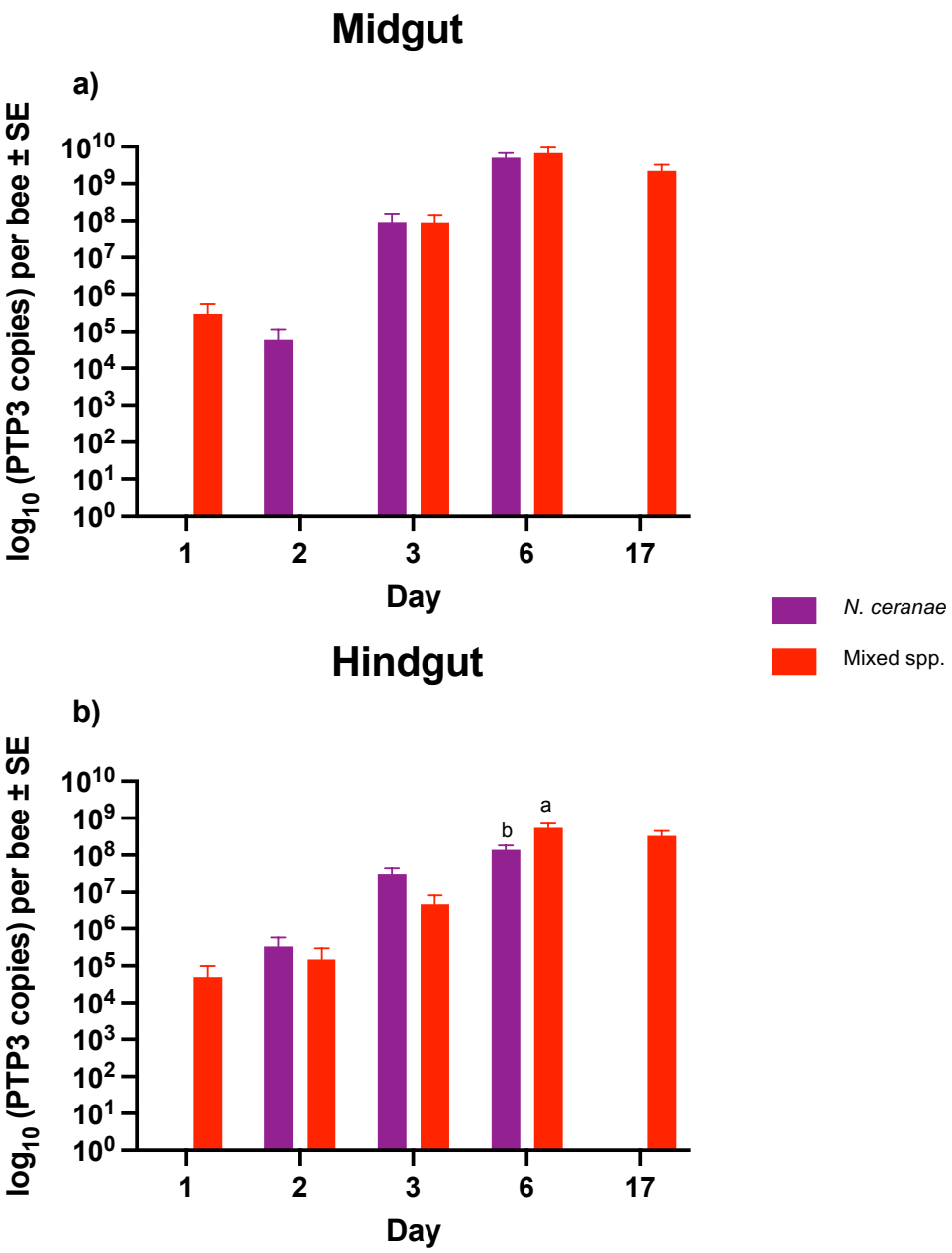


Figure 3.3 Mean (± SE) PTP3 expression (copies) in tissues by treatment for **a)** midgut, and **b)** hindgut at 1, 2, 3, 6, and 17 dpi. No data for *N. ceranae* only at 17 dpi due to all bees dying. Letters indicate statistically significant differences between treatments (α=0.05). See Supplementary Table 3.3 for statistical comparisons done using linear mixed effects models, one-way ANOVAs, or Kruskal-Wallis tests

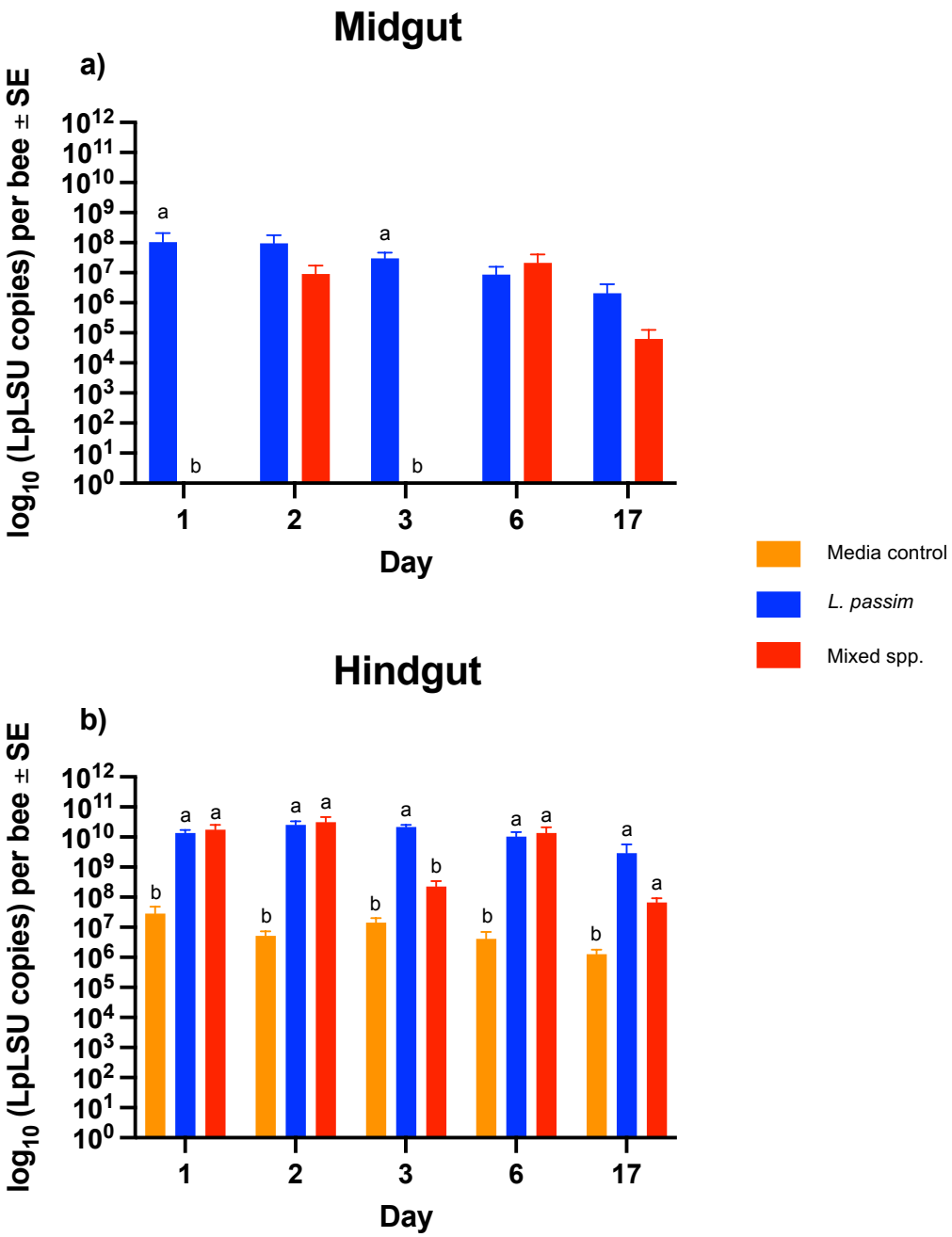


Figure 3.4 Mean (± SE) LpLSU expression (copies) in tissues by treatment for **a)** midgut, and **b)** hindgut at 1, 2, 3, 6, and 17 dpi. Letters indicate statistically significant differences between treatments (α=0.05). See Supplementary Table 3.3 for statistical comparisons done using linear mixed effects models, one-way ANOVAs, or Kruskal-Wallis tests

Chapter 4 Effects of *Nosema ceranae* and *Lotmaria passim* infections on honey bee foraging behaviour and physiology

4.1. Introduction

The Western honey bee (*A. mellifera* L) is one of the world's most intensively managed pollinators required for the pollination of many agricultural crops. This pollinator is infected by a variety of parasites and pathogens, such as *Nosema ceranae* and *Lotmaria passim*, that compromise bee health (Evans and Schwarz 2011). While there are a number of studies detailing the negative effects of *N. ceranae* and *L. passim* infections on individual honey bee physiology and behaviour, there are few that explore the relationship between parasite-induced physiological and behavioural changes, and even fewer exploring parasite-induced changes at the colony level (Goblirsch et al. 2013; Arismendi et al. 2020). Both *N. ceranae* and *L. passim* are common digestive tract parasites of the honey bee (Higes et al. 2006; Runckel et al. 2011; Williams et al. 2011; Morimoto et al. 2013; Ravoet et al. 2013; Schwarz et al. 2015; Holt and Grozinger 2016; Tritschler et al. 2017). *Nosema ceranae* is a microsporidian parasite that was described nearly a century after its congener, *N. apis* (Zander 1909), in 1996 (Fries et al. 1996), and is now considered to be the dominant *Nosema* spp. in areas where both are present (Higes et al. 2006; Chauzat et al. 2007; Paxton et al. 2007; Invernizzi et al. 2009; Currie et al. 2010; Runckel et al. 2011; Stevanovic et al. 2011; Traver and Fell 2011a; Morimoto et al. 2013; Emsen et al. 2016). *Lotmaria passim* (Schwarz et al. 2015) is a recently described trypanosomatid that was described much later than its relative, *Crithidia mellificae* (Langridge and McGhee 1967), and now outpaces *C. mellificae* in terms of infection prevalence (Runckel et al. 2011; Ravoet et al. 2013; Schwarz et al. 2015; Arismendi et al. 2016; Tritschler et al. 2017; Bartolomé et al.

2020). Despite the cosmopolitan distribution of both *N. ceranae* and *L. passim*, we still lack a comprehensive understanding of how both single and mixed infections of these parasites affect honey bee health, particularly at the colony level.

Within adult honey bees, *N. ceranae* infects midgut epithelial cells (Fries et al. 1996), degenerates midgut tissues (Dussaubat et al. 2012; Panek et al. 2018), and often leads to decreased lifespan (Higes et al. 2007; Goblirsch et al. 2013). This parasite is also associated with a number of physiological and behavioural changes in adult honey bees including altered immune responses, foraging behaviour, learning, and nursing ability (Higes et al. 2007; Antúnez et al. 2009; Goblirsch et al. 2013; Schwarz and Evans 2013; Li et al. 2018). The effect of *N. ceranae* on overall honey bee colony health is much more ambiguous, and appears to vary among geographic locations. In Germany, there is no apparent correlation between *N. ceranae* prevalence and colony mortality (Genersch et al. 2010; Gisder et al. 2010). In Spain, however, infection with *N. ceranae* is associated with decreases in colony size, brood-rearing capacity, honey production and ultimately colony collapse (Higes et al. 2008; Higes et al. 2009; Botías et al. 2013), often without observation of overt disease symptoms (Fernández et al. 2012). In Canada, increased *N. ceranae* spore abundance in spring is associated with increased colony mortality (Punko et al. 2021). In the United States, *N. ceranae* is only numerically more prevalent in colonies exhibiting colony collapse disorder (CCD) compared to non-CCD colonies (vanEngelsdorp et al. 2009), while metagenomic analyses shows that infection with either *N. ceranae* or *N. apis* was a differentiating factor between CCD-affected colonies and healthy colonies (Cox-Foster et al. 2007; vanEngelsdorp et al. 2009).

Lotmaria passim is found predominantly in the hindgut, with a preference for the distal portion of the ileum and anterior region of the rectum, near the papillae, within individual honey bees (Schwarz et al. 2015). The effects of this parasite on honey bee health at both the individual and colony level are poorly understood given its recent characterization. There are conflicting reports regarding the parasite's effect on honey bee lifespan, as infection can either negatively affect duration of life (Liu et al. 2019; Gómez-Moracho et al. 2020; Liu et al. 2020; Buendía-Abad et al. 2021), or have no effect (Arismendi et al. 2020). Within colonies, *L. passim* occurs concurrently with *N. ceranae* (Runckel et al. 2011; Ravoet et al. 2013; Tritschler et al. 2017), and while single infections with the former are correlated with increased winter colony mortality in Belgium (Ravoet et al. 2013), little is known about the impact of mixed infections at the colony level.

Honey bees exhibit age-based division of labour which is largely physiologically regulated by a feedback loop involving the yolk precursor protein ,vitellogenin (vg), and the endocrine factor juvenile hormone (Robinson and Vargo 1997; Amdam and Omholt 2003a; Guidugli et al. 2005; Goblirsch et al. 2013). Honey bees performing tasks within the colony such as nursing, and colony maintenance typically have decreased levels of juvenile hormone and increased levels of vg, and are younger in physiological and chronological age than those performing tasks outside the colony such as foraging, and guarding (Huang et al. 1994; Huang and Robinson 1996; Amdam and Omholt 2003a; Guidugli et al. 2005). Parasites can delay or advance physiological maturation in hosts (Beckage and Gelman 2004; Schafellner et al. 2007; Goblirsch et al. 2013; Wang et al. 2023). In honey bees, *N. ceranae* advances physiological maturation by increasing juvenile hormone levels and decreasing vg levels (Goblirsch et al.

2013). This physiological change can disrupt typical honey bee colony dynamics by altering honey bee behaviour, resulting in honey bees performing tasks (*e.g.,* foraging) that are physiologically advanced for their chronological age. Parasite-mediated changes in foraging behaviour can potentially result in less productive colonies, and as such necessitate changes in management recommendations for beekeepers.

Currently, there are no studies detailing the effects of both *N. ceranae* and *L. passim* in colonies under controlled inoculation conditions, which precludes us from fully understanding the pathology of these infections, and makes generating management recommendations for beekeepers difficult. Here, I investigate the physiological and behavioural changes of honey bees in colonies by experimentally infecting bees with both *N. ceranae* and *L. passim*. Specifically, I aim to determine whether locally-isolated parasites modify vitellogenin levels, and if these changes impact host behaviours such as first instance of foraging, average forager age, or foraging effort.

4.2. Methods and Materials

4.2.1 Nucleus colony preparation and inspection

Six, five-frame nucleus colonies were prepared 7-9 days in advance for each of the three replicates of this experiment that occurred during July and August of 2021 at Agriculture and Agri-Food Canada's (AAFC) Beaverlodge Research Farm (55°11'43.0"N; 119°17'57.3"W). Colonies were headed by locally-bred sister queens, and standardized with empty electron-beam irradiated drawn (empty) comb, frames containing honey and pollen, a frame of sealed brood belonging to the colony's queen, and adult bees of mixed ages from 5-7 non-experimental colonies. These non-experimental colonies were deemed to be free of *Varroa destructor* via alcohol washes (Fries et al. 1991), and devoid of detectable *N. ceranae*, *N. apis*, *L.*

passim, and *C. mellificae* via PCR. All colonies were provided with 1 L of 1:1 *(w/v)* sucrose, and half of a 15% pollen patty immediately after preparation and relocation to the experimental yard. The pollen patty was prepared by Global Patties (Airdrie, AB, Canada) according to their standard recipe to include 15% [by weight] pollen, 51% sucrose syrup, 18% distillers dried yeast, 16% defatted soy flour. Twenty-four hours in advance of a replicate of the experiment starting, and before foraging began, all supplemental feed was removed, and the six nucleus colonies were visually assessed for quantities of bees, brood, and food using the Liebefeld method (Imdorf 1987). Four of these six colonies were selected for the experiment. The experimental colonies were comprised of 2.8±0.1 frame sides of adult bees, 3.9±0.2 frame sides of honey/nectar, 0.3±0.04 frames sides of pollen, 2.4±0.1 frame sides of brood, and 3.3±0.2 frame sides of empty comb. On 7, 14, and 21 days after the initiation of the experiment, and after all observations and collections had concluded for the day, colonies were inspected for disease, and to verify experimental queens continued to egg-lay. No signs of disease were observed, and all experimental queens were maintained over all three replicates of the experiment.

4.2.2 Parasites

4.2.2.1 Lotmaria passim

An axenic culture of *L. passim* previously established at AAFC's Beaverlodge Research Farm in 2016 was grown to high density in Schneider's *Drosophila* medium supplemented with 10% fetal bovine serum, and 100 IU/mL Penicllin-100μg/mL Streptomycin-2.5μg/mL Amphotericin B at 25 ± 1.0°C (Schwarz et al. 2015) in a water-jacketed incubator (model 3326, Forma Scientific, Ottawa, ON, Canada). The culture was then cryopreserved in liquid nitrogen, and when needed, thawed and grown to high density in 15 and 50 mL centrifuge tubes under the above

conditions. Prior to inoculation, *L. passim* cultures were centrifuged at 200 × *g* for 10 min. After this initial centrifugation step, the supernatant was removed, and the pellet resuspended in 1 mL of 1:10 sucrose (50% *w/v*): 1× PBS solution. The resuspended pellet was then washed and centrifuged twice in 1 mL of 1:10 sucrose (50% *w/v*): 1× PBS solution at 200 × *g* for 10 min. After the final resuspension, a count was performed at 400× magnification using a counting chamber to estimate the number of motile, flagellated *L. passim* cells/mL of culture (MacInnis et al. 2023).

4.2.2.2 Nosema ceranae

Nosema ceranae spores were obtained from the midguts of *N. ceranae*-infected adult *A. mellifera* workers at AAFC's Beaverlodge Research Farm. The procedure for obtaining spores followed that from MacInnis et al. (2023), where cages of newly-emerged worker bees (NEBs) were inoculated with fresh *N. ceranae* spores, incubated for at least 14 days, and then dissected. After dissection, midguts were manually macerated in 1 mL of 1× PBS in a Stomacher® 80 Biomaster Standard Bag for 1 min before being macerated in a Stomacher® 80 blender for 5 min. The macerate was then passed through a 40 μm cell strainer (Cat# 352340, Fisher Scientific), and rinsed with 15 mL of 1× PBS. The resulting filtrate was then passed through a 10 μm separator (Cat# 60344, Pall Corporation, Ann Arbor, MI, USA) and rinsed with another 15 mL of 1× PBS. The 30 mL filtrate was then centrifuged at 800 × *g* for 10 min, and the pellet resuspended in 1 mL of 1× PBS. This 1 mL of *N. ceranae* spores in 1× PBS was treated with 100 IU/mL Penicillin-100 μg/mL Streptomycin (Cat# 15140122, Fisher Scientific, Ottawa, ON, Canada) for 1 hr to kill any contaminating bacteria (Schwarz and Evans 2013). After 1 hr, the *N. ceranae* spores were washed 3× in 1 mL of 1× PBS followed by centrifugation at 800 × *g*

for 10 min. After the final resuspension in 1 mL of 1× PBS, a count was performed at 400× magnification with a counting chamber to estimate the number of spores/mL (Cantwell 1970). *Nosema* spp. was verified as in MacInnis et al. (2023).

4.2.3 Experimental bees and paint-marking

One frame of eclosing worker bees was removed from each experimental colony <24 hr in advance of the experiment. Frames were maintained in incubators at 33 ± 1.0°C (models I36NLC8, I36NLC9, Percival Scientific, Perry, IA) until NEB collection occurred, and all NEBs used for the experiment were <24 hr old as in MacInnis et al. (2023). All NEBs were maintained in separate cages designated by source colony so that each colony would only receive manipulated NEBs originating from the same colony. NEBs from each colony were then further separated into two groups for paint-marking and inoculation such that each colony would receive two differently coloured paint-marked groups of NEBs which corresponded to their inoculation status (inoculated bee [IB] or background bee [BB]). Paint-marking was done by gently applying a dot of paint from non-toxic, water-resistant paint pens (uni POSCA® PC-5M's, and Craft Smart® medium line tip paint pens) to the thoraxes of NEBs. After marking, NEBs were placed into cages by paint colour such that each source colony had one cage of NEBs destined for inoculation (IB) and one cage of background bees (BB). Eight different colours / colour combinations were used so that each group of NEBs was identifiable within a replicate of the experiment.

4.2.4 Inoculation

NEBs from the inoculated group for each colony were individually inoculated with 5μL of one of the four following treatment groups diluted in 1:10 sucrose (50% *w/v*) : PBS (1×) solution via a 10 μL pipette. Treatment groups were as follows: 1) sucrose control (1:10 sucrose: 1×PBS), 2) *N.*

ceranae only (1.0×10^5 *N. ceranae* spores), 3) *L. passim* only (1.2×10^5 motile, flagellated *L. passim* cells), and 4) *N. ceranae* + *L. passim* (1.0×10^5 *N. ceranae* spores + 1.2×10^5 motile, flagellated *L. passim* cells). At the end of paint-marking and inoculation, each of the four colonies received their original frame of eclosing worker bees and any surplus bees not used for paint-marking and inoculation. Each colony then received their groups of paint-marked and/or inoculated NEBs (110 IB and 110 BB): 1) sucrose inoculated (IB) + sucrose background (BB), 2) *N. ceranae* inoculated (IB) + *N. ceranae* background (BB), 3) *L. passim* inoculated (IB) + *L. passim* background (BB), or 4) *N. ceranae* + *L. passim* inoculated (IB)+ *N. ceranae* + *L. passim* background (BB).

4.2.5 Foraging behaviour observations

After NEBs were marked and inoculated, they were returned to their respective colonies. To determine whether infection with *N. ceranae* and/or *L. passim* led to changes in foraging behaviour, such as first instance of foraging, average foraging age, or any shifts in resource collection (pollen or nectar), each colony was observed for 30 min every day from 7-21 days of age for IB and BB. Lower colony entrances were blocked with mesh hardware cloth, while upper entrances were blocked with a piece of duct tape during observation in order to record all returning paint-marked bees. The total number of paint-marked returning bees, the type of bees (IB or BB), and whether they were pollen or nectar foragers was recorded. Any returning paint-marked bees observed were collected and placed into perforated 50 mL centrifuge tubes for the duration of observation, and then released after observations were complete.

4.2.6 Sample collection for parasite quantification and quantification of vg

When honey bees were 7 and 14 days of age, four BB and four IB from each colony were randomly collected (before any foraging activity) and anaesthetized on dry ice to determine if

infection with *N. ceranae* or *L. passim* affected honey bee vg levels. Immediately after collection, the digestive tracts were removed from the IB and BB, placed in a 1.5mL microcentrifuge tube with sterile type I water, and macerated; carcasses of these bees (heads removed) were placed in new sterile 1.5 mL microcentrifuge tubes and immediately stored at -80°C until processing for vg expression occurred. A parasite count was then performed on the macerated digestive tracts as above to see if the bees were infected, and to determine the number of *N. ceranae* spores and motile, flagellated *L. passim* cells/mL in any infected bees. After counts were complete, samples were stored in 70% ethanol for long-term storage at -20°C.

At the four first instances of foraging (first four returning paint-marked bees) that occurred for each colony during daily colony observation, four paired samples of IB and BB were collected (*e.g.*, if a BB was foraging at a colony, an IB was collected from the colony after observation was done to complete the pair) and anaesthetized on dry ice. These samples were then processed for parasite presence, intensity and stored for vg expression processing as described above.

4.2.7 Sample collection and processing for foraging effort

On the last day of the experiment when IB and BB were 21 days of age, as many returning paint-marked foragers as possible were collected from each colony onto dry ice during and after the daily 30 min observation period. These returning marked foragers were placed into individual 1.5 mL centrifuge tubes and stored at -80°C until processing occurred. Processing involved regurgitating any nectar loads, and removing pollen loads from the corbiculae of frozen foragers, and weighing the loads to the nearest 0.1mg.

4.2.8 RNA extraction and cDNA synthesis

Total RNA was extracted from IB and BB carcasses using TRIzol and Phasemaker tubes (Cat # 15596018, A33248, Invitrogen, ThermoFisher Scientific, Ottawa, ON, Canada) following the manufacturer's protocol after being flash-frozen and ground in liquid nitrogen. Following extraction, the purified RNA pellets were resuspended in nuclease-free water as per the manufacturers protocol, and then quantity and quality checked using a BioTek Epoch spectrophotometer and Take3 microvolume plate (Agilent, Santa Clara, CA, USA). Following quantification, 400ng of total RNA were aliquoted for DNA degradation using DNAse I (RNAse-free) (Cat# AM2224, Ambion, Life Technologies, ThermoFisher Scientific, Ottawa, ON, Canada), followed by first strand cDNA synthesis using SuperScript II Reverse Transcriptase and RNaseOUT (Cat # 18064071, 10777019, Invitrogen, ThermoFisher Scientific, Ottawa, ON, Canada) following the manufacturer's protocol. Random hexamers (100ng) and oligo $(dT)_{12\text{-}18}$ (50ng) (Cat # N8080127, 18418012, Invitrogen, ThermoFisher Scientific, Ottawa, ON, Canada) were used to prime transcription. After transcription was complete, cDNA was diluted 1:5 in nuclease-free water. The absence of contaminating gDNA in the cDNA was confirmed via endpoint PCR with reference primer pairs targeting honey bee ribosomal protein (Rp) S5 (RpS5). For each batch of transcribed samples, we had aliquots of samples (1-2) that both received and did not receive reverse transcriptase. Each endpoint PCR reaction consisted of: 1× PCR buffer, 0.4mM dNTPs, 0.25μM of forward and reverse primer (see Supplementary Table 4.1), 0.625U Taq, 1.5μL cDNA template, and nuclease-free water up to a final volume of 25μL. Thermalcycler settings were as follows: 97°C for 5 min followed by 35 cycles of 95°C for 30 sec, 54°C for 30 sec, 72°C for 30 sec, a final extension step of 72°C for 7 min, and a hold at 4°C until stopped (ProFlex PCR System, Applied Biosystems, ThermoFisher Scientific, Ottawa, ON, Canada). PCR products

were visualized on a 2% agarose gel stained with SYBR®Safe DNA Gel Stain (Cat # S33102, Invitrogen, ThermoFisher Scientific, Ottawa, ON, Canada) at 100 V for 40 min.

4.2.9 RT-qPCR for absolute quantification of vg levels

Primers used in this study can be found in Supplementary Table 4.1, and include pairs targeting RpS5, β-actin, and vg. Data were normalized to RpS5 only as it proved to be stable across all treatments and timepoints, while β-actin was not stable across all treatments at one time point. qRT-PCR analyses were performed in in 384-well clear/white plates with Microseal 'B' adhesive seals using the Bio-Rad CFX384 real time system (Cat # HSP3805, MSB1001, Bio-Rad, Mississauga, ON, Canada). Each plate was comprised of 32 cDNA samples assessed for all three targets (RpS5, β-actin, vg) in parallel. No template control (NTC) reactions for each target were run on each plate to monitor contamination, and positive controls for each target were included on each plate using serial dilutions (2.00×10^1-2.00×10^7 copies) of 499bp synthetic gene fragments (gblocks®; Integrated DNA Technologies, Coralville, IA, USA) (Supplementary Table 4.2) to monitor amplification efficiency, primer efficiency, and to generate standard curves for each target (Supplementary Table 4.1). All samples were run in duplicate or triplicate, and the protocol and analysis for this portion of the study were based on recommended guidelines (Bustin et al. 2009). All reactions contained equal amounts of template cDNA (2μL of 1:5 diluted cDNA, 1:50 diluted cDNA for samples requiring further dilution, or nuclease-free water for NTCs), 200nM each of a forward and reverse primer, and 1× SsoAdvanced Universal SYBR® Green Supermix (Cat # 1725274, Bio-Rad, Mississauga, ON, Canada). Thermalcycler conditions were as follows: 97°C for 2 min, followed by 40 cycles of 95°C for 2 sec, 60°C for 5 sec, and melt curve analysis from 65-95°C at 0.5°C / 5 sec increments to confirm expected dissociation curves.

All RT-qPCR experiments and analyses were handled by the same individual to minimize any potential handler variation.

The average copy number / bee for the target gene (vg) was calculated for each bee, and then the average copy number of vg / bee for each bee was normalized to its corresponding average RpS5 value, normalized to the average number of copies of RpS5 across all bees:

$$\left(\frac{Average\ Vg\ copies/bee}{Average\ RpS5\ copies/bee}\right) * (Average\ copies\ of\ RpS5/all\ bees^{\dagger})$$

†= Average copies of RpS5 / all bees per tissue type and time point being analyzed.

4.2.10 Statistical Analyses

All statistical analyses were performed in 'R' v. 4.2.1 "Funny Looking Kid" within 'R'Studio v. 2022.07.1+554 "Spotted Wakerobin" for Mac OS X (R et al. 2022).

4.2.10.1 Foraging Behaviour

To determine if there was an effect of parasite treatment and/or time on the returning number of observed total foragers, nectar foragers, and pollen foragers during the 14 days of observation, I compared the ratios of IB:BB for each forager type and parasite treatment × time combination. I also investigated whether there was an effect of parasite treatment on returning number of observed total foragers, nectar foragers, and pollen foragers for each day of observation by comparing the IB:BB ratios. Ratios were used rather than total numbers of IB:BBs in these comparisons to eliminate any colony effects. To calculate the ratios, I adjusted all observed values by adding +1 to each to address situations in which there were no returning foragers observed for either the IB or BB groups of a treatment before calculating the IB:BB ratio. Treatments that had no foragers observed for both IB and BB groups were not adjusted (i.e., a true zero). For each forager type and parasite treatment × time combination, ratios were

compared using linear mixed effects models (lmer, 1.1-31 lme4) with replicate as a random effect, and parasite treatment and time as fixed effects. Model reduction was performed by removing the least significant fixed effect first, and then comparing the original and new models with the ANOVA function (anova). Model fit was assessed by plotting the scaled residuals, examining Levene's test for homogeneity of variance, and examining the Kolmogorov-Smirnov's test and Shapiro-Wilk's test for overdispersion (simulateResiduals, 0.4.6, DHARMa and shapiro.test(resid(), R Core Team 2022). In order to achieve adequate model fit, the total pollen foragers dataset was Box-Cox transformed (λ=0.5, squareroot transform) (boxcox, 7.3-57, MASS) prior to models being run. Additionally, the random effect of replicate for this dataset was also very small (zero), so it was removed and a one-way ANOVA (aov) followed by multiple comparisons was performed (glht, 1.4-20, multcomp), rather than a linear mixed effect model.

To determine if there was an effect of parasite treatment on returning number of observed total, pollen, and nectar foragers for each day of the experiment, I compared IB:BB ratios using linear mixed effects models with replicate as a random effect, and parasite treatment as a fixed effect. The significance of the fixed effect was evaluated using an *F*-test (Anova, 3.1-0, car), and multiple comparisons were performed as above. Model fit was assessed as above. In order to achieve adequate model fit, some datasets were Box-Cox transformed prior to models being run, and ANOVA tables being generated. In some of the models generated, random effects were very small (or zero), so the random effect of replicate was removed, and a one-way ANOVA (aov) performed. In both instances, multiple comparisons were performed as above when necessary. One dataset could not be analyzed using linear mixed effects models or one-way ANOVA due to the presence of zeroes, and in this case data

were analyzed using a Kruskal-Wallis rank sum test followed by Dunn's test of multiple comparisons (dunn.test, 1.3.5, dunn.test).

4.2.10.2 Forager age

Average forager age was calculated by summing the total foragers observed per inoculation type (IB or BB) per treatment for each day foragers were observed, and then multiplying each sum by the day they were observed (i.e., 5 control BB foragers observed on day 17 = 5×17); this value was then divided by the total number of foragers observed per inoculation type per treatment during the entire observation period. Differences in average forager age within treatments (IB vs BB) were compared using linear mixed effects models with parasite treatment as a fixed effect, and replicate as a random effect, or one-way ANOVAs with parasite treatment as the fixed effect when the random effect of replicate was very small. Model fit was assessed as above in 4.2.10.1. Ratios of average forager age (IB:BB, without any adjustment) were compared among treatments using a linear mixed effects model (parasite treatment as the fixed effect, and replicate as the random effect) followed by multiple comparisons of means as above in 4.2.10.1 to determine if there was an overall effect of parasite treatment on average forager age.

4.2.10.3 vg quantification

To determine if there was an effect of parasite treatment on vg expression, the ratios of IB:BB were calculated for each treatment as above, and compared at each of the three sampling collections (when IB and BB were 7 and 14 days of age, as well as at first instance of foraging). Ratios were compared rather than absolute quantities of vg to account for any colony effects as mentioned previously. All data were analyzed using linear mixed effects models with treatment as a fixed effect, and replicate as a random effect, or one-way ANOVAs with treatment as a fixed

effect, when the random effect of replicate was very small (or zero) in the linear mixed effect models (i.e., when IB and BB were 7 days of age). Model fit was assessed as above in 4.2.10.1, and Box-Cox transformations were applied as necessary.

I also evaluated whether there was an effect of parasite inoculation on vg expression at the first instance of foraging by comparing the vg expression of IB and BB within each parasite treatment (e.g., *N. ceranae* IB vs *N. ceranae* BB). Additionally, I also compared vg expression of foraging and non-foraging (colony-collected) paint-marked bees within each parasite treatment collected at the first instance of foraging to see if foraging status (foraging and non-foraging) affected vg expression. All data were analyzed using linear mixed effects models with treatment (parasite treatment or foraging status respectively), as the fixed effect and replicate as the random effect or one-way ANOVA with treatment as the fixed effect when the random effect was found to be very small in the linear mixed effects models. Data were Box-Cox transformed if necessary, and model fit assessed as above in 4.2.10.1.

4.2.10.4 Parasite quantification

Density of *N. ceranae* spore and motile, flagellated *L. passim* cell loads were compared between *N. ceranae* or *L. passim* only infections ,and mixed *N. ceranae* + *L. passim* infections for IB and BB collected at 7 and 14 days of age, and at the first instances of foraging using Welch's Two-Sample t-test (t.test).

4.2.10.4 Foraging effort

Pollen and nectar load IB:BB ratios were calculated as above in 4.2.10.1 (with +1 adjustment to replicate averages to address situations in which there were no loads within a replicate) to eliminate any colony effects, and then compared to determine if there was an effect of treatment on foraging effort (load size). Both pollen and nectar load ratio data were analyzed

using linear mixed effects models or one-way ANOVAs with parasite treatment as the fixed effect and replicate as the random effect (linear mixed effects models). Data were Box-Cox transformed as necessary, and model fit assessed as above in 4.2.10.1.

4.3 Results

4.3.1 Foraging Behaviour

There was an additive effect of treatment and day on total returning forager ratios (χ^2_3=20.9, *P*<0.001, χ^2_1=23.6, *P*<0.001 respectively) and total returning nectar forager ratios observed (χ^2_3=9.86, *P=0.02*, χ^2_1=28.8, *P*<0.001 respectively), but only an effect of parasite treatment on returning pollen forager ratios observed ($F_{3,68}$=3.95, *P*<0.001). Parasite treatment influenced the ratio of total returning foragers on observation days 19 (χ^2_3=91.2, *P*<0.001) and 20 (χ^2_3=12.6, *P*<0.001, Fig. 4.1, Supplemental Table 4.3). Bees inoculated with both *N. ceranae* and *L. passim* were foraging at a significantly higher rate than bees from all other treatments on day 19 and at a significantly higher rate than bees from the control treatment on day 20. On day 17 bees from the mixed infection treatment were foraging for pollen at a significantly higher rate than control or *N. ceranae*-only inoculated bees (χ^2_3=11.4, *P*<0.001), and on day 18 were foraging at a significantly higher rate than bees from all other treatments (χ^2_3=11.1, *P*<0.001, Fig. 4.1b, Supplemental Table 4.4). On day 19, bees from the mixed infection treatment were also foraging for nectar at a significantly higher rate than all other treatments (χ^2_3=12.3, *P*<0.001, Fig. 4.1c , Supplemental Table 4.5). There was also a marginal statistically significant effect of parasite treatment on average forager age ratios (χ^2_3=7.52, *P*=0.057, Fig. 4.2a), but there was a significant effect of parasite treatment on average forager age for bees within the mixed

infection treatment, whereby inoculated bees began foraging earlier than uninoculated bees by 0.6 days (χ^2_1=7.03, *P*<0.001, Fig. 4.2b), Supplemental Table 4.6).

4.3.2 vg quantification

There was no effect of parasite treatment on vg expression for IB:BB for bees at either 7 days ($F_{3,8}$=0.689*, P*=0.584, Fig. 4.3a, Supplemental Table 4.7) or 14 days of age ($F_{3,8}$=5.802*, P*=0.122 Fig. 4.3b, Supplemental Table 4.7), or at first instance of foraging ($F_{3,8}$=3.155, *P*=0.086, Fig. 4.3c, Supplemental Table 4.7).

There were, however, differences in vg expression for bees by inoculation type (IB vs BB) for all three parasite treatments at first instance of foraging (*N. ceranae* only: χ^2_1=3.82*, P*=0.051, *L. passim* only: χ^2_1=6.56*, P*=0.011, *N. ceranae* + *L. passim*: χ^2_1=38.9*, P*<0.001, Fig. 4.4a, Supplemental Table 4.8) but not the control (χ^2_1=1.09*, P*=0.299). There were also differences in vg expression for foraging vs. non-foraging bees across all four treatments (control: χ^2_1=7.39*, P*<0.001, *N. ceranae* only: $F_{3,22}$=36.6*, P*<0.001, *L. passim* only: χ^2_1=25.7*, P*<0.001, *N. ceranae* + *L. passim*: χ^2_1=14.8*, P*<0.001, Fig. 4.4b , Supplemental Table 4.9).

4.3.3 Parasite quantification

There was no difference in motile, flagellated *L. passim* cell densities for bees collected from the *L. passim* and mixed infection treatments at 7 (*t=0.7446*, df=2, *P*=0.495, Fig. 4.5a) and 14 days of age (*t=0.9916*, df=9, *P*=0.347, Fig. 4.5b), as well as at first instance of foraging (*t=1.128*, df=21, *P*=0.209, Fig. 4.5c). Though there were no differences in *N. ceranae* spore densities for bees collected from the *N. ceranae* and mixed infection treatments at 7days of age (*t=1.895*, df=21, *P*=0.495, Fig. 4.5a), there were differences in densities for bees collected at 14 days of age (*t=4.191*, df=22, *P*<0.001, Fig. 4.5b), and at first instance of foraging (*t=2.578*, df=25,

P=0.016, Fig. 4.5c) when bees from the mixed infection treatment had significantly more spores than bees from the *N. ceranae* treatment.

Lastly, there were no differences in ratios of pollen and nectar loads (IB:BB) by weight across all four treatments (pollen: $F_{3,4}$=0.924, *P*=0.472, nectar: χ^2_3=0.10, *P*=0.992, Supplemental Table 4.10) on the last day (day 21) of the experiment.

4.4 Discussion

This is the first study to examine the effects of local isolates of *N. ceranae* and *L. passim* on honey bee foraging behaviour and physiology at the colony level under controlled inoculation conditions. Overall, I found that there was an additive effect of parasite treatment and time on the total forager and total nectar forager ratios observed, likely driven by the increasing number of bees foraging over time (especially bees from the mixed infection treatment) as they transitioned physiologically from in-colony tasks to tasks outside the colony, such as foraging. For ratios of returning total pollen foragers observed, we found only an effect of treatment, and no effect of time. This was caused by bees inoculated with mixed infections foraging significantly more than control bees, or bees inoculated with *N. ceranae* only. For individual days of observation, I found that infection status had a significant effect on the total number (relative to BB) of returning foragers on days 17, 18, 19, and 20. Bees inoculated with mixed infections consistently foraged more frequently than bees from the control, or *N. ceranae* only groups. These results demonstrate that there is a clear effect of the mixed infection on honey bee foraging behaviour. These findings also demonstrate that infections decrease vg levels, such that bees infected with *N. ceranae*, *L. passim* alone, or in mixed infections, are effectively physiologically advanced for their chronological age. This physiological change is particularly

pronounced for bees inoculated with the mixed infection as well as *L. passim* alone, and accounts for the observed behavioural changes and altered colony foraging dynamics for these parasitized bees.

Given that parasites possess the ability to modify host physiology and behaviour (Thompson and Kavaliers 1994; Poulin 2010), it is highly probable that I observed more bees infected with *L. passim* foraging because of the parasite modifying bee behaviour to facilitate its transmission. As *L. passim* is found predominantly in the honey bee hindgut, with a strong preference for the anterior rectum and distal portion of the ileum (Schwarz et al. 2015), it is possible that it could be transmitted horizontally via a faecal-oral route at flowers. This is one mechanism by which the related parasite, *Crithidia bombi* Lipa and Triggiani, is horizontally transmitted. Foraging bumblebees infected with *C. bombi* defecate on flowers, leaving behind infective cells to be horizontally transmitted to other foraging bees (Durrer and Schmid-Hempel 1994; Figueroa et al. 2019).

These *L. passim*-infected bees could also be attempting to self-medicate by foraging strongly for plant compounds with high antimicrobial activity. Plant compounds such as eugenol, carvacrol, and cinnamaldehyde reduce *L. passim* cell densities in infected honey bees in cages (Palmer-Young et al. 2022). Honey bees infected with *N. ceranae* prefer honey with high antibiotic activity, (Gherman et al. 2014), while honey bee colonies infected with *Ascosphaera apis* increase resin collection in response to infection (Simone-Finstrom and Spivak 2012). Additionally, honey bees infected with *N. ceranae* and *L. passim* (alone or mixed) have increased responsiveness to sucrose compared to uninfected bees (MacInnis et al. 2023), suggesting that foraging can offset the energetic costs of infection.

Though bees inoculated with *L. passim* (single or mixed) had the highest returning forager ratios at each instance of observation, bees from at least one parasitized group had higher returning forager ratios than the control at each observation. This suggests that parasitism affects foraging rate, but not foraging effort, as we observed no differences in the weights of pollen and nectar loads across treatments.

Though a treatment effect on returning forager ratios was not always observed, and a lack of treatment effect was shown for average foraging age ratios, it is possible that treatment effects might be seen on these measurements more clearly in resource-limited environments *i.e.,* condition-dependent virulence (Brown et al. 2000; Mayack and Naug 2009; Naug and Gibbs 2009). Although treatment did not effect average forager age ratios, it is interesting to note that inoculated bees from the mixed infection treatment began foraging at a significantly younger age (18.9 days of age) than BB (19.5 days of age) from the same treatment.

No differences in vg expression were observed for IB:BB at any of the three sampling points (7 and 14 days of age, and at first instance of foraging). However, when IB and BB vg expression was compared within treatments at first instance of foraging, I found that IB in each treatment (except for the control) had significantly lower vg expression than their BB counterparts. Additionally, IB from the mixed infection treatment had the lowest vg expression out of all groups examined. Reductions in vg expression due to parasitic infection in honey bees has been observed on several occasions (Goblirsch et al. 2013; Arismendi et al. 2020; Liu et al. 2020), and has been associated with decreased nutritional status and lifespan, as well as precocious foraging (Guidugli et al. 2005; Goblirsch et al. 2013; Higes et al. 2013; Arismendi et al. 2020). Within the mixed infection treatment group in this study, I observed not only

precocious foraging, but an increase in foraging rate overall, and very pronounced differences in vg levels between IB and BB, illustrating that parasite treatment can affect both behaviour and physiology.

At the first instance of foraging, I also separated the bees by foraging status (foraging or non-foraging [within colony bees] rather than IB vs BB), and observed a similar but more significant trend in regard to vg expression. Foraging bees (43/48 were IB) had significantly lower vg levels than non-foraging bees (41/48 were BB, of the 7 BB, 5 were from the control treatment) counterpart for each treatment. This finding is expected, as honey bees exhibit age polyethism, which is in part physiologically controlled by a feedback loop involving vg and juvenile hormone. Honey bees performing tasks within a colony typically have higher levels of vg and lower levels of juvenile hormone, while honey bees performing tasks outside the colony such as foraging, typically have lower levels of vg and higher levels of juvenile hormone (Huang et al. 1994; Amdam and Omholt 2003a; Guidugli et al. 2005; Goblirsch et al. 2013). Because most of the foraging bees were also IB rather than BB, this further illustrates that infection can affect physiology.

When parasite densities were quantified from bees sampled at 7 and 14 days of age, and first instance of foraging, a trend similar to the one observed with vg expression in IB vs BB emerged. When bees were 7 days of age, *L. passim* cell densities were similar for bees infected with *L. passim* only or *N. ceranae* + *L. passim*, while bees inoculated with *N. ceranae* only had higher spore densities (numerically) compared to bees inoculated with both *N. ceranae* + *L. passim*. However, at 14 days of age, and at first instance of foraging bees inoculated with both *N. ceranae* + *L. passim* had higher parasite densities compared to bees inoculated with only *N.*

ceranae or *L. passim*. Bees inoculated with both *N. ceranae* + *L. passim* had a significantly higher density of spores compared to those inoculated with only *N. ceranae*, and had (numerically) more *L. passim* cells compared to bees inoculated with only *L. passim*. (Tritschler et al. 2017) observed a positive correlation between *N. ceranae* and *L. passim* densities in field-collected honey bees infected with both parasites. MacInnis et al. (2023) also observed an increase in parasite density (numerically) for bees inoculated with both parasites

The Increased foraging, decreased vg levels, and increased parasite density observed particularly for bees co-infected with *N. ceranae* + *L. passim* in this study illustrate that effects of *N. ceranae* + *L. passim* can alter bee behaviour and physiology. It may also be plausible that bees are able to compensate for the negative effects of infection (*e.g.*, decreased lifespan or perceived colony decline) via behavioural changes that may affect physiology (ability to immunomodulate), such as increased foraging, or the consumption of greater quantity and quality resources when available, which has been previously documented in Hymenoptera (Moret and Schmid-Hempel 2000; Basualdo et al. 2014; Kay et al. 2014; Jack et al. 2016).

This study illustrates that, under controlled inoculation conditions, infection with *N. ceranae* and *L. passim* affects honey bee physiology and behaviour. Honey bees inoculated with *L. passim* (either alone or with *N. ceranae*) had higher returning forager ratios than control honey bees, or honey bees inoculated only with *N. ceranae*. Additionally, bees inoculated with any of the parasite treatments also had significantly reduced vg expression relative to uninoculated bees within the same treatment. This is a physiological change that can be associated with precocious foraging, which is what I observed in the mixed infection treatment. Honey bees with mixed infections also had higher densities of *L. passim* compared with honey

bees inoculated with only *L. passim*, and significantly higher densities of *N. ceranae* spores compared to honey bees inoculated with only *N. ceranae*. As such, beekeepers are recommended to monitor their colonies for *N. ceranae* and *L. passim*, as reduced vg expression and early onset of foraging can disrupt typical colony dynamics. This could lead to smaller, less-productive colonies, and reduced income for beekeepers. Additionally, novel parasite management strategies should be explored, particularly for *L. passim*.

4.5 Figures and Tables

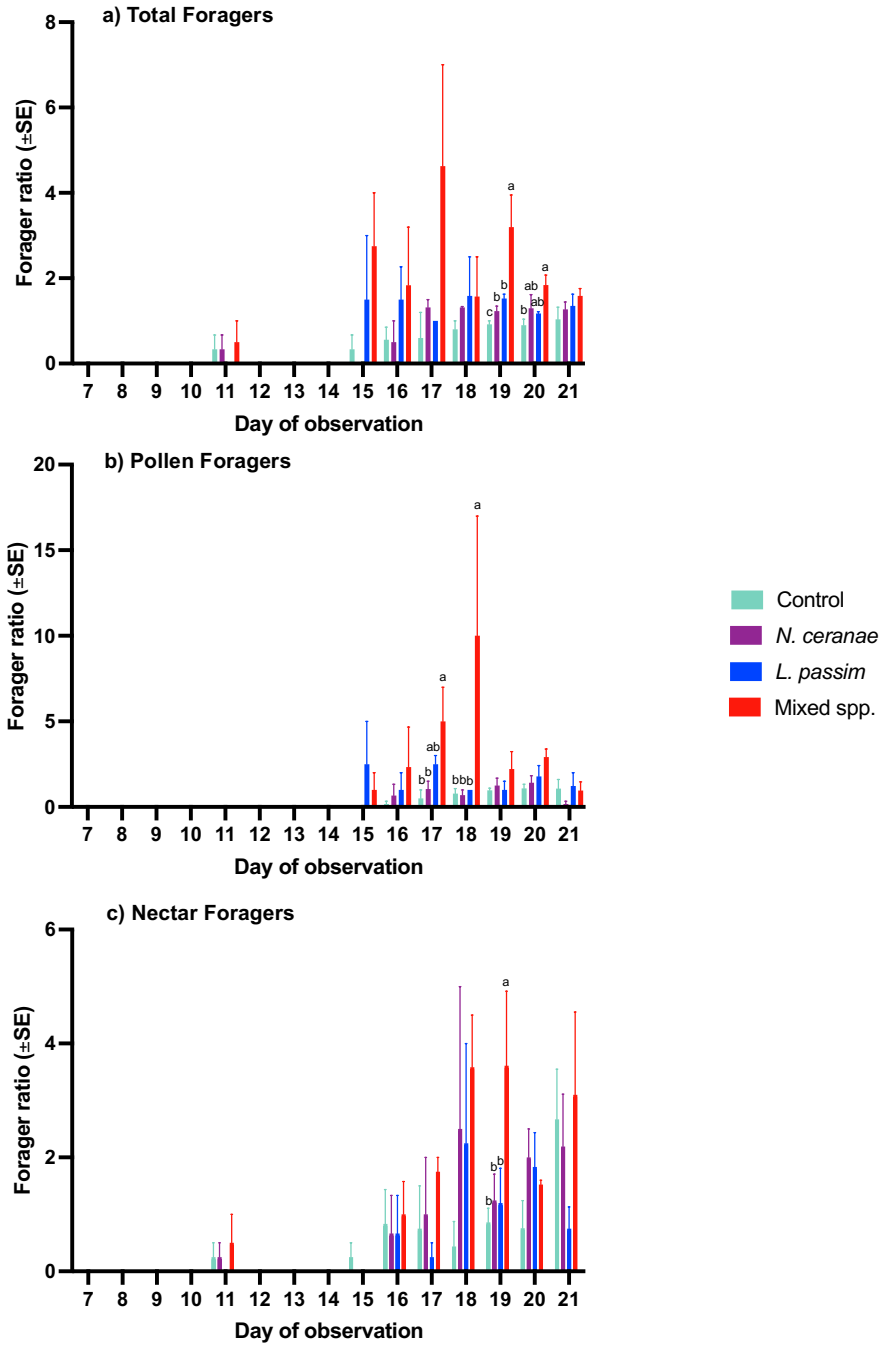


Figure 4.1 Ratios (mean ± SE) of IB (inoculated bee): BB (background bee) foragers observed during daily 30 min observation periods from days 7-21 of the experiment. For each day of observation, treatments are in the following order: Control, *N. ceranae*, *L. passim*, and Mixed spp. (bees inoculated with both *N. ceranae* and *L. passim*). Different letters indicate statistically significant differences between treatments at a given time point (lmer, aov, dunn.test;α=0.05, statistical comparisons and n values found in Supplementary Tables 4.3-4.5).

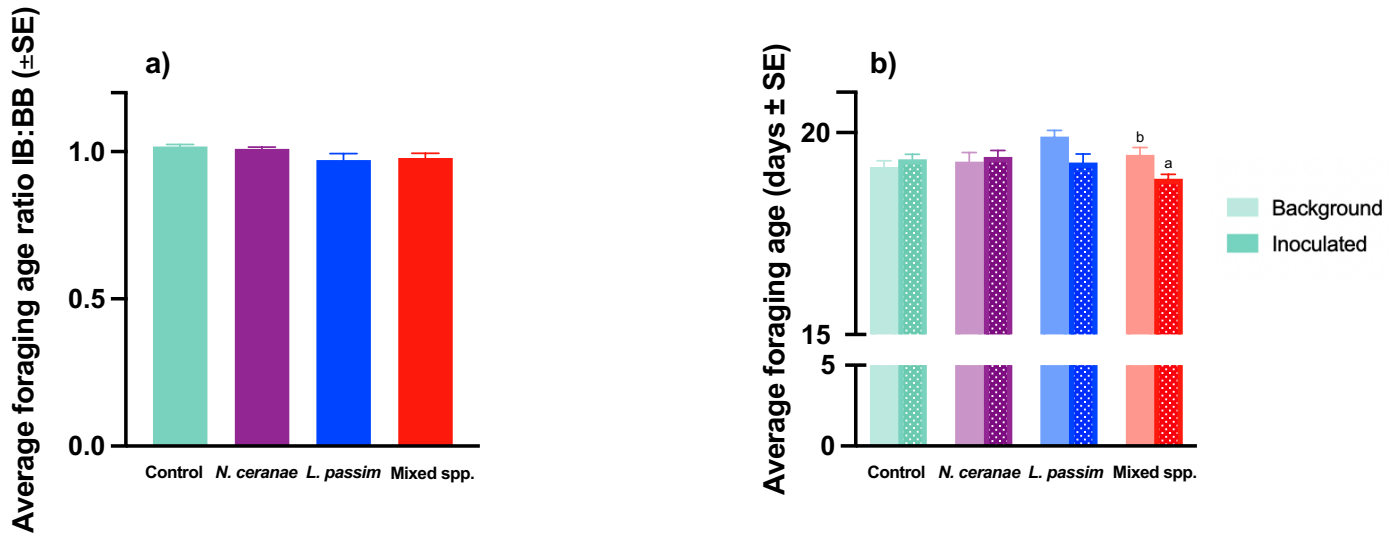


Figure 4.2 a) Average foraging age ratio of IB (inoculated bee) : BB (background bee) by parasite treatment group. **b)** Average foraging age of IB and BB by parasite treatment group. Light bars represent BB while dark bars represent IB. Mixed spp. refers to bees inoculated with both *N. ceranae* and *L. passim.* Letters indicate statistically significant differences between IB and BB bees within a treatment (lmer, aov; α=0.05, statistical comparisons and n values found in Supplementary Table 4.6).

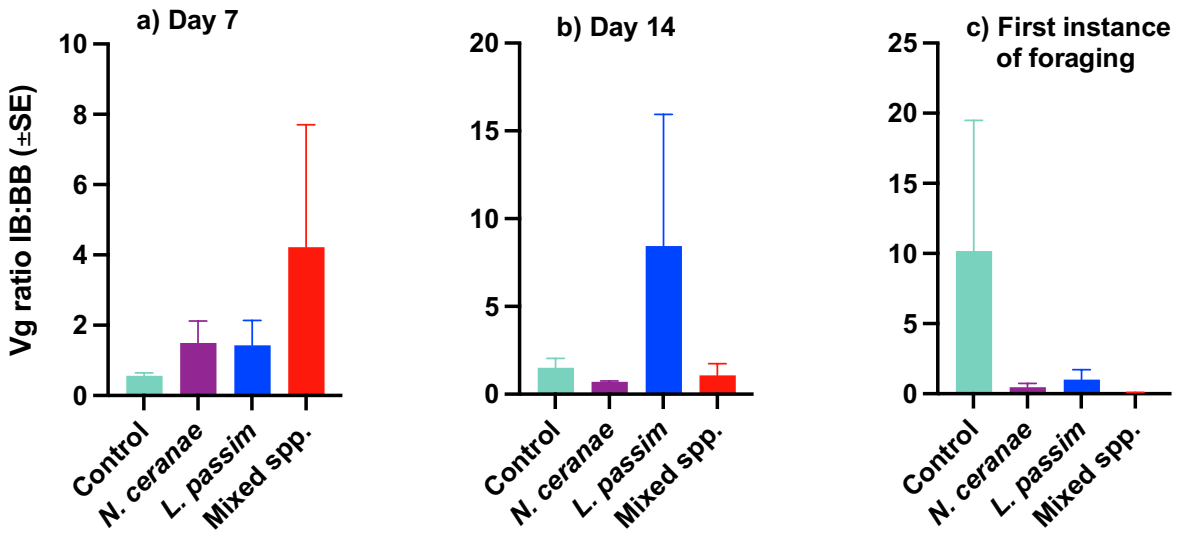


Figure 4.3 Ratios of IB (inoculated bee): BB (background bee) vitellogenin (vg) levels from bees collected at day 7, day 14, and at first instance of foraging during the experiment. Mixed spp. refers to bees inoculated with both *N. ceranae* and *L. passim.* No statistically significant differences were detected between treatments (lmer, aov; α=0.05, statistical comparisons and n values found in Supplementary Table 4.7).

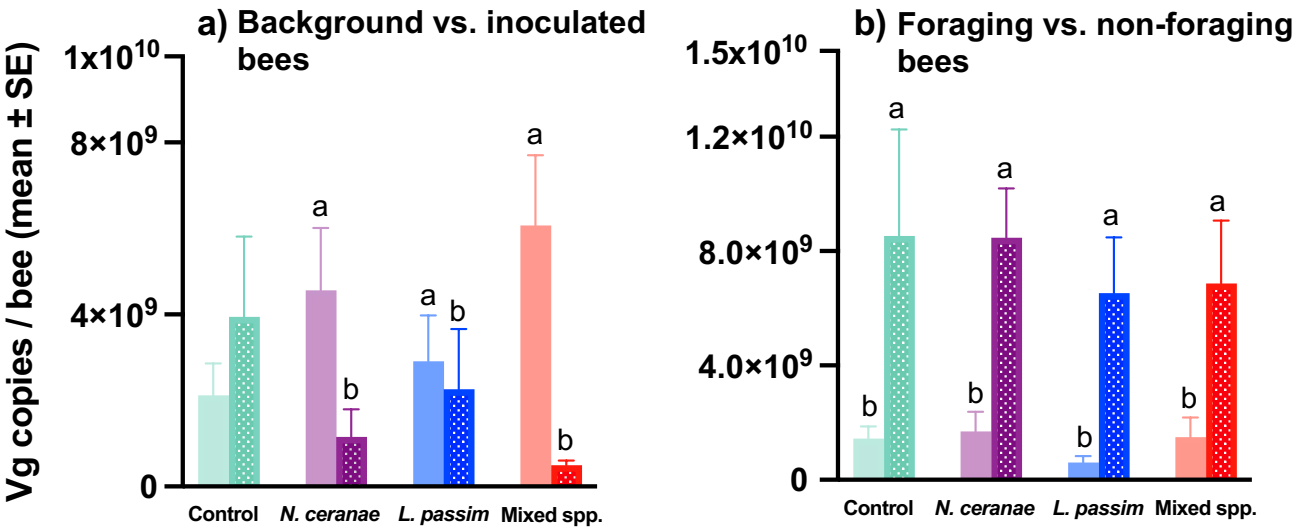


Figure 4.4 a) Vitellogenin (vg) copies per bee collected at first instance of foraging by inoculations status (inoculated bee [IB] or background bee [BB]) per treatment. Light bars represent BB while dark bars represent IB. **b)** Vg copies per bee collected at first instance of foraging by foraging status (foraging, not foraging) per treatment. Light bars represent foraging bees while dark bars represent not foraging bees. Mixed spp. refers to bees inoculated with both *N. ceranae* and *L. passim.* Letters indicate statistically significant differences between IB and BB bees within a treatment, and foraging and not foraging bees within a treatment (lmer, aov; α=0.05, statistical comparisons and n values found in Supplementary Table 4.8 and Supplementary Table 4.9 respectively).

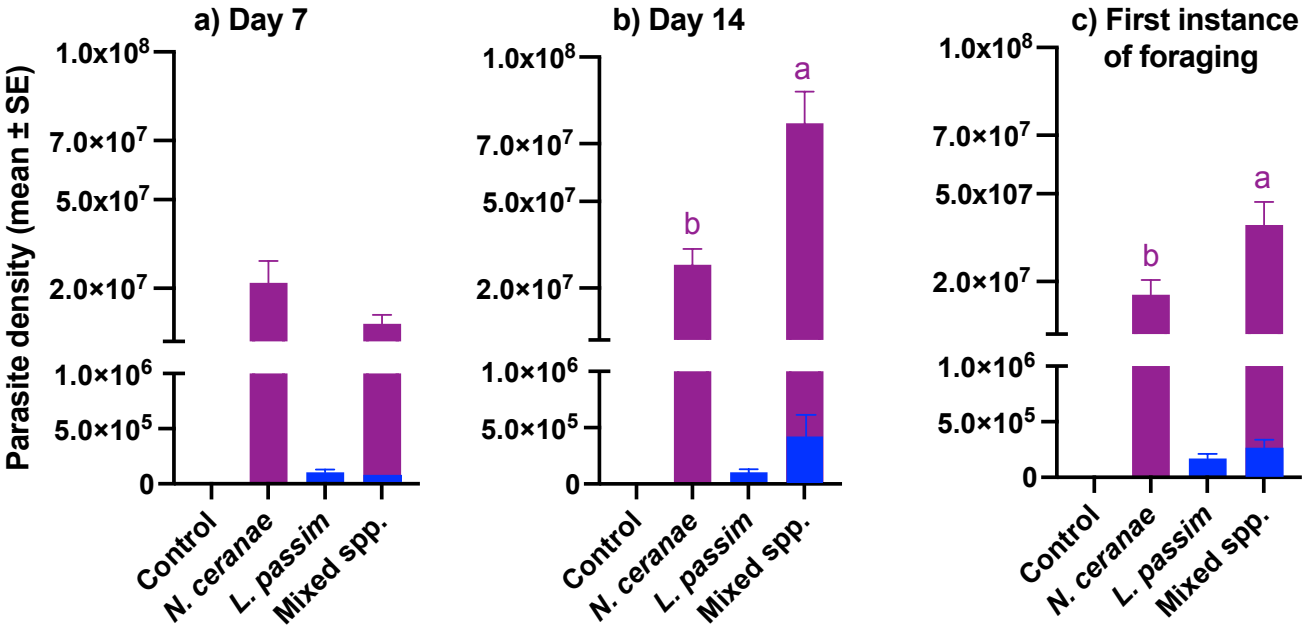


Figure 4.5 *Nosema ceranae* and *L. passim* densities in bees collected at day 7, day 14, and first instance of foraging during the experiment. **a)** Parasite densities at day; 7 **b)** Parasite densities at day 14; **c)** Parasite densities at first instance of foraging. Mixed spp. refers to bees inoculated with both *N. ceranae* and *L. passim.* Purple bars represent *N. ceranae* densities in single and mixed infections, and purple letters indicate statistically significant differences in *N. ceranae* densities between single and mixed infections. Blue bars represent *L. passim* densities in single and mixed infections (t.test; α=0.05).

Chapter 5 Summary and synthesis

5.1 General discussion

The Western honey bee (*A. mellifera*) is one of the world's most intensively managed pollinators required for the pollination of many agricultural crops. Unfortunately, this pollinator is plagued by a variety of parasites and pathogens, including the two commonly encountered digestive tract parasites, *Nosema ceranae* and *Lotmaria passim* (Evans and Schwarz 2011). Despite their cosmopolitan distribution, and that honey bees can be co-infected with both parasites (Tritschler et al. 2017; Williams et al. 2021), we still lack a comprehensive understanding of how *N. ceranae* and *L. passim* affect honey bee health at both the individual and colony level. The objective of this thesis was to assess the effects of both single and mixed *N. ceranae* and *L. passim* infections on honey bee survival, behaviour, and physiology at both the individual and colony level. This was done to determine if these infections, particularly those involving *L. passim*, require monitoring and management by the beekeeping industry to improve honey bee health.

The sucrose responsiveness assay and survival curve experiment conducted in chapter 2 illustrated that infection in general affects honey bee responsiveness to sucrose, and lifespan. Infected bees had increased responsiveness to sucrose compared to uninfected bees, suggesting that infected bees experience higher levels of hunger due to increased energetic stress caused by the presence of parasites. Infected bees also had lower survival compared to uninfected bees. Interestingly, bees infected with both parasites had increased survival compared to bees infected with *N. ceranae* only, while bees infected with *L. passim* had only slightly lower survival compared to controls, despite there being no statistical differences in

parasite densities across treatment groups. This suggests that *L. passim* is not as virulent as *N. ceranae*, and that there may be an interaction occurring between the two parasites.

In chapter 3, I found that in general, honey bees did not mount distinct, localized humoral defense responses to infection with *N. ceranae* or *L. passim* with the 3 AMPs I quantified at five time points. This could be because honey bees respond systemically rather than locally to these infections, or because they respond locally, but with AMPs I did not quantify. It is also possible that I did not detect any differences in AMP expression because I missed events, such as cell rupturing, that elicited ephemeral AMP expression. I also found, based on LpLSU expression in midgut and hindgut tissues from this experiment, that *L. passim* does appear to have a preference for hindgut tissues, which has been suggested previously (Schwarz et al. 2015; Buendía-Abad et al. 2022). Rapid increases in PTP3 expression were also observed in both midgut and hindgut tissues over the course of this experiment, and I suggested that high expression in the hindgut tissues could indicate that *N. ceranae* is able to reproduce there, particularly in the ileum which is directly attached to the midgut via the pylorus. This could explain the elevated mortality of honey bees infected with only *N. ceranae* in chapter 2. Few differences in parasite densities across treatment groups were noted, similar to chapter 2, indicating that if there are any interactions occurring between the two parasites, it is likely not a resource competition interaction, especially given *L. passim*'s purported preference for hindgut tissue.

The increased survival of bees infected with both *N. ceranae* and *L. passim* compared to *N. ceranae*, alone, in chapter 2 may be explained by combining the findings of both chapters 2 and 3. Given the lack of differences in parasite densities between single and mixed *N. ceranae*

and *L. passim* infections in chapters 2 and 3, the minimal effect *L. passim*-only infections had on lifespan in chapter 2, and *L. passim*'s observed preference for the hindgut in chapter 3, it is possible that *L. passim* is performing a function similar to the bumblebee gut microbiome, which protects bumble bees from infection against the trypanosomatid *Crithidia bombi* (Koch and Schmid-Hempel 2011; Koch and Schmid-Hempel 2012). The bumble bee gut microbiota forms layers that line the hindgut portion of the digestive tract (ileum and rectum) (Hammer et al. 2021). *Lotmaria passim* has also been observed to form a single layer covering epithelial cells in the ileum and rectum (Buendía-Abad et al. 2022). The honey bee also has gut microbiota largely confined to the hindgut that could play a role in the biosynthesis of nutrients (Kwong and Moran 2016). It is conceivable that if *N. ceranae* could reproduce within the hindgut (particularly the ileum) that a layer of *L. passim* may reduce or prevent *N. ceranae* infection in this region, allowing the bee to continue performing essential functions, such as biosynthesizing nutrients, which would contribute to an increased lifespan.

In chapter 4, I evaluated the effects of single and mixed *N. ceranae* and *L. passim* infections on bee behaviour and physiology. I found that infection had no effect on foraging effort, but that it did significantly reduce vitellogenin (vg) expression at the first instance of foraging, with bees infected with both parasites having the lowest vg expression. Interestingly, bees infected with both *N. ceranae* and *L. passim* also had a significantly younger average forager age compared to uninoculated bees, and had increased parasite densities at day 14 and first instance of foraging compared to infections with *N. ceranae* (significantly increased) and *L. passim* only (numerically increased). Increased parasite density in the mixed infection bees suggests that an interaction may be occurring between the two parasites, and supports the

finding from chapters 2 and 3, which is that if an interaction is occurring, that it is not a resource competition interaction. The decrease in vg expression and average foraging age for bees infected with both parasites, indicates that these parasites can affect bee behaviour and physiology. These particular changes could disrupt colony dynamics, leading to smaller, less-productive colonies.

Collectively, the results of this thesis suggest that *N. ceranae* and *L. passim* do require monitoring and management by the beekeeping industry. In chapter 2, results showed that in individual bees, the two parasites negatively affected honey bee lifespan, and increased responsiveness to sucrose. In chapter 3, I showed that bees did not respond locally to infection with *N. ceranae* and *L. passim*, and suggested that the two parasites are not competing for resources. Finally, chapter 4 illustrated that *N. ceranae* and *L. passim* have the ability to disrupt bee physiology and behaviour at the colony level. Based on these findings, and the cosmopolitan distribution of *L. passim*, beekeepers should routinely monitor for this parasite. Because this parasite has a preference for the honey bee hindgut, and is likely transmitted via a fecal-oral route, monitoring could be conducted by beekeepers in early spring and fall when they are also monitoring for *N. ceranae*. Early spring and fall are recommended as there is little population turnover into the fall, and honey bees are largely confined to the colony over winter months in temperate climates preventing them from taking cleansing flights to defecate and eliminate any parasites. Because of this, parasite density would be high at these time points, allowing beekeepers to determine whether they have the parasite or not.

5.2 Future work

How single infections of *L. passim,* and concurrent infections of *N. ceranae* and *L. passim*, affect the survival of different honey bee stocks should be carefully investigated. This is because choosing a resistant or tolerant stock is an expedient way for beekeepers to promote colony health, which could result in increased productivity and survival. We should also investigate whether bees have distinct, ephemeral systemic responses to *N. ceranae* and *L. passim*, as understanding how bees respond to these infections could inspire novel management techniques. Finally, the impact of *N. ceranae* and *L. passim* on honey production, brood-rearing capacity, and colony survival should be evaluated. If decreases in productivity and survival are observed, as I predict, monitoring and management strategies will need to be employed.

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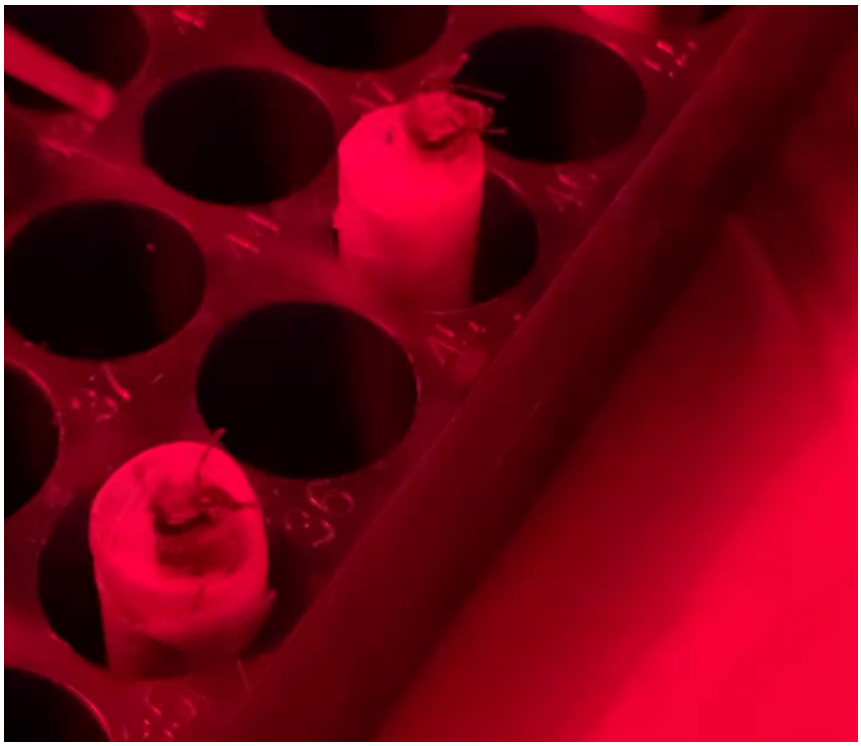
Appendix A. Supplementary Information for Chapter 2



Supplementary Figure 2.1. Newly-emerged bees (NEBs) prepared in harnesses (cut-off portions of drinking straws and parafilm) for the sucrose responsiveness assay.

Supplementary Table 2.1. Amplified Cytochrome b product sequenced for species identification

Organism	Target	Product sequence	Identity (%)	Accession number
L. passim	Cytochrome b	TCGTGTAAAGCGGAGAAAGAAGAAAAGGCTTTTAACGT CAGGTTGCTTATTAAGAGTATATGGAGTAGGTTTTAGTT TAGGTTTTTTTATATGCATGCAAATTATATGTGGTGTATG TTTAGCATGATTATTTTTTAGCTGTTTTATATGTACTAATT GATATTTTGTTTTATTTTTATGAGATTTTGATTTAGGTTTT GTAATACGAAGTGCACATATATGCTTTACATCATTATTAT TCTTTTTACTGTATGTTCATATATTTAAAGCGATCGTTTTA ATAATTTTATTTGATACTCATATTTTAGTATGAGCAGTAG GTTTTATCATATATATATTCATAGTAGTTATAGGTTTTATT GGATATGTATTACCATGTACAATGATGTCTTATTGAGGTC TAACTGTTTTTAGTAATATTTTAGCAACAGTACCAGTTATT GGTGTTTGGCTATGTTATTGAATATGAGGTAGTGAGTTTA TAAATGATTTTACACTATTAAAATTACATGTGCTACATGTA TTATTGCCATTTGTTTTAATATTAGTTATAGTTATGCACTT ATTTTGCTTACATTATTTTATGAGCTCGGATGGTTTTTGT GATCGTTTTGCTTTTTATTGTGAACGTTTGTGT	98.69	KM980180.1

Supplementary Table 2.2. Number of newly-emerged bees (NEBs)/cage/treatment/replicate in the survival curve experiment

Replicate	Treatment	Cage A INOC	Cage A UNINOC	Cage B INOC	Cage B UNINOC
1	Control	50	54	51	49
1	Media control	52	56	-	-
1	*N. ceranae* only	55	43	52	49
1	*L. passim* only	-	-	50	48
1	*N. ceranae* + *L. passim*	52	50	44	47
2	Control	51	45	54	42
2	Media control	-	-	-	-
2	*N. ceranae* only	54	44	50	48
2	*L. passim* only	44	48	-	-
2	*N. ceranae* + *L. passim*	52	52	-	-
3	Control	54	44	53	51
3	Media control	49	46	54	46
3	*N. ceranae* only	-	-	49	50
3	*L. passim* only	-	-	52	57
3	*N. ceranae* + *L. passim*	-	-	-	-
4	Control	51	48	51	47
4	Media control	-	-	48	49
4	*N. ceranae* only	47	47	54	47
4	*L. passim* only	50	46	-	-
4	*N. ceranae* + *L. passim*	53	49	48	48

INOC= inoculated NEBs

UNINOC = Uninoculated NEBs for social interaction

-= Not monitored due to mortality that occurred which was not due to experimental infection

Supplementary Table 2.3. qPCR primers, and standard curve information used to determine *Lotmaria passim* infection status in survival curve and sucrose responsiveness NEBs as well as *L. passim* loads in sucrose responsiveness NEBs in this study

Assay	Primer	Sequence	NT	Tm (°C)	Size	Melt temp (°C)	R^2	Efficiency (%)	Slope	*Y*-int	Reference
RpS5 qPCR	RpS5 F	AATTATTTGGTCGCTGGAATTG	22	51.6	115	77.5	0.998	96.5	-3.409	38.857	Evans *et al.* 2006
	RpS5 R	TAACGTCCAGCAGAATGTGGTA	22	55.9							
L. passim cytb qPCR	Lpcytb_F2	AGTATGAGCAGTAGGTTTTATTATA	25	49.3	146	75.5	0.994	86	-3.699	37.973	Vejnovic *et al*. 2018
	Lpcytb_R	GCCAAACACCAATAACTGGTACT	23	55.4							

Supplementary Table 2.4. Confirmation of infection and parasite densities in newly-emerged bees (NEBs) from the sucrose responsiveness assay. After the sucrose responsiveness assay was complete, all responding NEBs were individually frozen in 1.5mL microfuge tubes and stored at -20°C until processing occurred. At processing, 4 (or 5) NEBs from each treatment in both 2019 and 2021 were randomly chosen to confirm infection status and parasite density via microscopy (*N. ceranae*) and qPCR (*L. passim*)

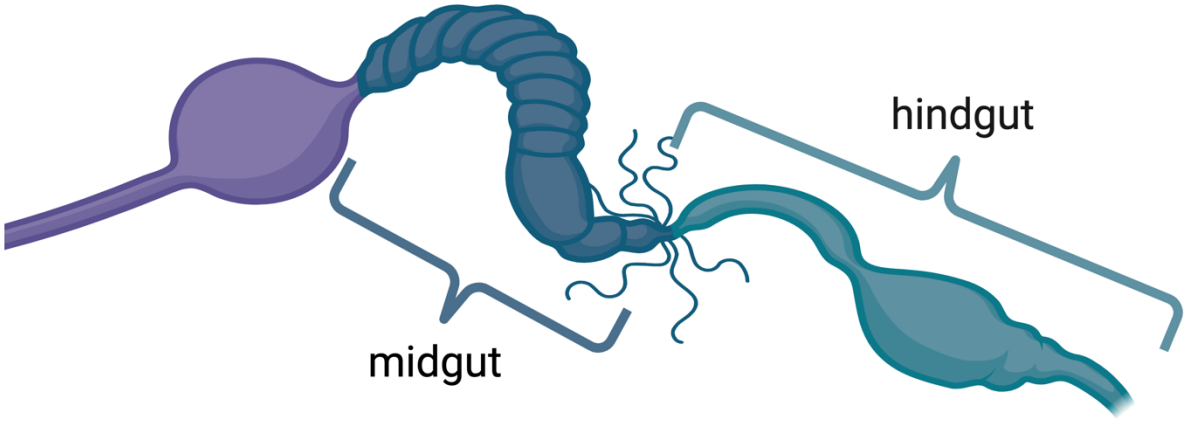
Year	Trt	ID#	*N.c*	*N.a*	*L.p*	*N. ceranae* spores/bee	cytb copies/bee NORM
2019	CTRL	1	-	-	-	0	0
2019	CTRL	2	-	-	-	0	0
2019	CTRL	3	-	-	-	0	0
2019	CTRL	4	-	-	-	0	0
2019	MEDIA	5	-	-	-	0	0
2019	MEDIA	6	-	-	-	0	0
2019	MEDIA	7	-	-	-	0	0
2019	MEDIA	8	-	-	-	0	0
2019	NC	9	+	-	-	46500000	0
2019	NC	10	+	-	-	76500000	0
2019	NC	11	+	-	-	85750000	0
2019	NC	12	+	-	-	73500000	0
2019	LP	13	-	-	+	0	1643336.103
2019	LP	14	-	-	+	0	3664332.509
2019	LP	15	-	-	+	0	2472.551
2019	LP	16	-	-	+	0	3189343.126
2019	LPNC	17	+	-	+	80250000	203398.569
2019	LPNC	18	+	-	+	77750000	6993867.836
2019	LPNC	19	+	-	-	70750000	0
2019	LPNC	20	+	-	+	97000000	1033041.891
2021	CTRL	21	-	-	-	0	0
2021	CTRL	22	-	-	-	0	0
2021	CTRL	23	-	-	-	0	0
2021	CTRL	24	-	-	-	0	0

2021	MEDIA	25	-	-	-	0	0
2021	MEDIA	26	-	-	-	0	0
2021	MEDIA	27	-	-	-	0	0
2021	MEDIA	28	-	-	-	0	0
2021	NC	29	+	-	-	89000000	0
2021	NC	30	+	-	-	100250000	0
2021	NC	31	+	-	-	78000000	0
2021	NC	32	+	-	-	92000000	0
2021	LP	33	-	-	+	0	653.337
2021	LP	34	-	-	+	0	1332.646
2021	LP	35	-	-	-	0	0
2021	LP	36	-	-	+	0	2183.409
2021	LPNC	37	+	-	+	73750000	21299.723
2021	LPNC	38	+	-	+	183250000	83279.668
2021	LPNC	39	+	-	+	86250000	363157.514
2021	LPNC	40	+	-	+	94750000	159154.591
2021	LP	41	-	-	+	0	3292.705

Trt=Treatment, ID#=Bee ID, NC= *N. ceranae*, LP=*L. passim*, +=positive for a parasite, -=negative for a parasite, cytb copies/bee NORM = *L. passim* cytb copies/bee normalized to RpS5

Appendix B. Supplementary Information for Chapter 3

Supplementary Figure 3.1. Image of the honey bee digestive tract depicting the tissues that constituted midgut and hindgut sections used in this experiment.



Supplementary Table 3.1. Endpoint, qPCR primers and standard curve information used in this study to verify *Nosema* spp., and *L. passim*, and quantify immune gene expression levels in collected bees

Assay	Primer	Sequence	NT	Tm (°C)	Size	Melt temp (°C)	R^2	Efficiency (%)	Slope	*Y*-int	Reference
RpS5 endpoint and qPCR	RpS5 F	AATTATTTGGTCGCTGGAATTG	22	51.6	115	78-78.5	0.989	91.5	-3.545	42.121	Evans *et al.* 2006
	RpS5 R	TAACGTCCAGCAGAATGTGGTA	22	55.9							
N. ceranae endpoint	NoscRNAPol F2	TGGGTTCCCTAAACCTGGTGGTTT	24	64.0	662	-	-	-	-	-	Gisder *et al.* 2013
	NoscRNAPol R2	TCACATGACCTGGTGCTCCTTCT	23	63.5							
N. apis endpoint	NosaRNAPol F2	AGCAAGAGACGTTTCTGGTACCTCA	25	63.7	297	-	-	-	-	-	Gisder *et al.* 2013
	NosaRNAPol R2	CCTTCACGACCACCCATGGCA	21	65.0							
N. ceranae PTP3 qPCR	qPCR-Nc83-F	AGCACAAGGAGTCGAGCAAA	20	59.9	100	80-80.5	0.995	96.5	-3.410	43.202	Rodríguez-García *et al.* 2018
	qPCR-Nc83-R	TGCTGCCTCAAATCCTACCT	20	58.7							
LpLSU qPCR	LpLSU-F	GTGCAGTTCCGGAGTCTTGT	20	57.4	103	84.5-85.5	0.993	90.2	-3.582	44.304	Vanengelsdorp *et al.* 2009
	LpLSU-R	CTGAGCTCGCCTTAGGACAC	20	57.5							
Apidaecin qPCR	Apid-F	TTTTGCCTTAGCAATTCTTGTTG	23	52.6	81	79.5-80.5	0.997	89.5	-3.602	43.353	Boncristiani *et al.* 2012
	Apid-R	GTAGGTCGAGTAGGCGGATCT	21	57.7							
Defensin-1 qPCR	Def1-F	TGCGCTGCTAACTGTCTCAG	20	57.1	119	81.5-82	0.999	90.8	-3.564	41.700	Evans 2006
	Def1-R	AATGGCACTTAACCGAAACG	20	53.7							
Hymenoptaecin qPCR	Hym-F	CTCTTCTGTGCCGTTGCATA	20	55.2	200	82.5	0.998	90.7	-3.568	41.547	Evans 2006
	Hym-R	GCGTCTCCTGTCATTCCATT	20	55.0							

Supplementary Table 3.2. Target genes and sequences used in this study to create standard curves, and determine gene expression levels in bees

Organism	Target	Sequence	Amplicon length	% identity	Accession number
Apis mellifera	RpS5	**TAACGTCCAGCAGAATGTGGTAAATATTTT GCATTTTTCTCTTTAACGGCAATATAATCT TGTAAAGACATATCATTCACTTGTACATCA TCACAATTCCAGCGACCAAATAATT**	115	100	XM_006570236
Apis mellifera	Apidaecin	**TTTTGCCTTAGCAATTCTTGTTGTTACCTT TGTAGTCGCGGTATTTGGGAATACCAACCT AGATCCGCCTACTCGACCTAC**	81	100	NM_001011642
Apis mellifera	Defensin-1	**AATGGCACTTAACCGAAACGTTTGTCCCAG AGATCTTTGAAACTGGTTTTTCGACAAATA CAAACTCCTTTCTCGCAATGACCTCCAGCT TTACCCAAACTGAGACAGTTAGCAGCGCA**	119	100	FJ546136
Apis mellifera	Hymenoptaecin	**GCGTCTCCTGTCATTCCATTCTTATCGTAG ACACGTTGTTTGTAATCAATGTCCAAGGAT GGACGACTTTTTCCTTCTTTAGTTCCTTGA ATGACAATGGATCCTCTTTCTTGTCGTCGG AAACGAGTCGGGATATAATCCATTGTATCC TCAGGTTCCAATTCCGCTTGAGCAGAAACG TATGCAACGGCACAGAAGAGAA**	200	99.5	FJ546166
Nosema ceranae	PTP3	**AGCACAAGGAGTCGAGCAAAGTGCTCAAAA TGAAATACAACACAGAATGGATTTGGCTGA TGCAATGAAAGAAAATGCTAAGGTAGGATT TGAGGCAGCA**	100	100	XM_024473556

Lotmaria passim	LpLSU	**GTGCAGTTCCGGAGTCTTGTTTCGAGACAT CTGCCAGATGGGGAGTTTGT-TGGGGCGGC ATATCTGTTACACGACAACGCAGGTGTCCT AAGGCGAGCTCAG**	103	98.1	CP140162

Supplementary Table 3.3. Mean copies±SE per bee for each gene of interest per day per tissue type and treatment. Bold letters indicate statistically significant differences between treatments at a given time point (*P<0.05*)

Treatment	Gene	Day	Tissue type	Mean copies ± SE	n	Statistical comparison
Sucrose CTRL	Apidaecin	1	Midgut	0±0	5	
Media CTRL	Apidaecin	1	Midgut	$6.896\times10^{5}\pm6.896\times10^{5}$	7	
N. ceranae only	Apidaecin	1	Midgut	$1.518\times10^{7}\pm1.123\times10^{7}$	7	χ^2_4=11.249, *P*=0.024
L. passim only	Apidaecin	1	Midgut	$6.152\times10^{6}\pm4.223\times10^{6}$	7	
N. ceranae + *L. passim*	Apidaecin	1	Midgut	$1.303\times10^{6}\pm1.123\times10^{6}$	8	
Sucrose CTRL	Apidaecin	2	Midgut	$1.475\times10^{6}\pm1.013\times10^{6}$	7	
Media CTRL	Apidaecin	2	Midgut	$5.825\times10^{5}\pm5.825\times10^{5}$	8	
N. ceranae only	Apidaecin	2	Midgut	$3.270\times10^{6}\pm1.313\times10^{6}$	6	χ^2_4=9.9161, *P*=0.042
L. passim only	Apidaecin	2	Midgut	$2.628\times10^{5}\pm1.840\times10^{5}$	8	
N. ceranae + *L. passim*	Apidaecin	2	Midgut	$3.062\times10^{6}\pm1.431\times10^{6}$	6	
Sucrose CTRL	Apidaecin	3	Midgut	$1.976\times10^{6}\pm16.192\times10^{5}$**ab**	8	
Media CTRL	Apidaecin	3	Midgut	0±0**b**	7	
N. ceranae only	Apidaecin	3	Midgut	$2.913\times10^{7}\pm1.658\times10^{7}$**a**	7	χ^2_4=12.566, *P*=0.014
L. passim only	Apidaecin	3	Midgut	$2.428\times10^{7}\pm2.215\times10^{7}$**ab**	6	
N. ceranae + *L. passim*	Apidaecin	3	Midgut	$7.699\times10^{6}\pm3.494\times10^{6}$**ab**	8	
Sucrose CTRL	Apidaecin	6	Midgut	$2.645\times10^{7}\pm1.233\times10^{7}$	7	
Media CTRL	Apidaecin	6	Midgut	$1.056\times10^{7}\pm6.120\times10^{6}$	8	
N. ceranae only	Apidaecin	6	Midgut	$1.121\times10^{7}\pm7.353\times10^{6}$	8	χ^2_4=4.4703, *P*=0.346
L. passim only	Apidaecin	6	Midgut	$6.541\times10^{6}\pm3.247\times10^{6}$	8	
N. ceranae + *L. passim*	Apidaecin	6	Midgut	$3.354\times10^{7}\pm2.279\times10^{7}$	8	
Sucrose CTRL	Apidaecin	17	Midgut	$1.216\times10^{7}\pm8.797\times10^{6}$	7	
Media CTRL	Apidaecin	17	Midgut	$2.129\times10^{8}\pm1.401\times10^{8}$	8	
N. ceranae only	Apidaecin	17	Midgut	-‡	-	χ^2_3=3.977, *P*=0.264
L. passim only	Apidaecin	17	Midgut	$1.488\times10^{7}\pm1.264\times10^{7}$	8	
N. ceranae + *L. passim*	Apidaecin	17	Midgut	$3.760\times10^{7}\pm3.436\times10^{7}$	8	

Supplementary Table 3.3 cont'd

Treatment	Gene	Day	Tissue type	Mean copies ± SE	n	Statistical comparison
Sucrose CTRL	Apidaecin	1	Hindgut	$9.940\times10^{8}\pm2.904\times10^{8}$	5	
Media CTRL	Apidaecin	1	Hindgut	$5.841\times10^{8}\pm1.386\times10^{8}$	7	
N. ceranae only	Apidaecin	1	Hindgut	$1.669\times10^{9}\pm5.287\times10^{8}$	7	$F_{4,29}$=2.716, P=0.049
L. passim only	Apidaecin	1	Hindgut	$9.342\times10^{8}\pm6.263\times10^{8}$	7	
N. ceranae + *L. passim*	Apidaecin	1	Hindgut	$1.220\times10^{9}\pm2.320\times10^{8}$	8	
Sucrose CTRL	Apidaecin	2	Hindgut	$8.144\times10^{8}\pm2.244\times10^{8}$**a**	7	
Media CTRL	Apidaecin	2	Hindgut	$1.123\times10^{8}\pm4.299\times10^{7}$**b**	8	
N. ceranae only	Apidaecin	2	Hindgut	$2.029\times10^{8}\pm5.722\times10^{7}$**b**	6	χ^2_4=18.372, P=0.001
L. passim only	Apidaecin	2	Hindgut	$4.422\times10^{8}\pm2.489\times10^{8}$**ab**	8	
N. ceranae + *L. passim*	Apidaecin	2	Hindgut	$1.849\times10^{8}\pm4.996\times10^{7}$**b**	6	
Sucrose CTRL	Apidaecin	3	Hindgut	$4.702\times10^{8}\pm1.775\times10^{8}$	8	
Media CTRL	Apidaecin	3	Hindgut	$6.449\times10^{7}\pm1.410\times10^{7}$	8	
N. ceranae only	Apidaecin	3	Hindgut	$2.603\times10^{8}\pm3.598\times10^{7}$	7	$F_{4,33}$=2.808, P=0.041
L. passim only	Apidaecin	3	Hindgut	$6.607\times10^{8}\pm3.274\times10^{8}$	7	
N. ceranae + *L. passim*	Apidaecin	3	Hindgut	$2.355\times10^{8}\pm3.956\times10^{7}$	8	
Sucrose CTRL	Apidaecin	6	Hindgut	$7.702\times10^{8}\pm2.538\times10^{8}$	8	
Media CTRL	Apidaecin	6	Hindgut	$2.390\times10^{8}\pm1.170\times10^{8}$	8	
N. ceranae only	Apidaecin	6	Hindgut	$7.434\times10^{8}\pm4.351\times10^{8}$	8	$F_{4,35}$=1.279, P=0.297
L. passim only	Apidaecin	6	Hindgut	$4.745\times10^{8}\pm1.048\times10^{8}$	8	
N. ceranae + *L. passim*	Apidaecin	6	Hindgut	$3.051\times10^{8}\pm8.092\times10^{7}$	8	
Sucrose CTRL	Apidaecin	17	Hindgut	$1.374\times10^{9}\pm6.517\times10^{8}$	7	
Media CTRL	Apidaecin	17	Hindgut	$9.062\times10^{8}\pm3.548\times10^{8}$	8	
N. ceranae only	Apidaecin	17	Hindgut	-‡	-	$F_{3,24}$=0.135, P=0.938
L. passim only	Apidaecin	17	Hindgut	$1.406\times10^{9}\pm8.284\times10^{8}$	6	
N. ceranae + *L. passim*	Apidaecin	17	Hindgut	$3.116\times10^{9}\pm2.278\times10^{9}$	7	

Supplementary Table 3.3 cont'd

Treatment	Gene	Day	Tissue type	Mean copies ± SE	n	Statistical comparison
Sucrose CTRL	Hymenoptaecin	1	Midgut	0±0**ab**	5	
Media CTRL	Hymenoptaecin	1	Midgut	$4.620\times10^{5}\pm3.160\times10^{5}$**ab**	7	
N. ceranae only	Hymenoptaecin	1	Midgut	$1.023\times10^{6}\pm4.707\times10^{5}$**a**	7	χ^2_4=10.577, *P*=0.032
L. passim only	Hymenoptaecin	1	Midgut	$1.795\times10^{7}\pm1.782\times10^{7}$**ab**	7	
N. ceranae + *L. passim*	Hymenoptaecin	1	Midgut	0±0**b**	8	
Sucrose CTRL	Hymenoptaecin	2	Midgut	$1.044\times10^{5}\pm1.044\times10^{5}$	7	
Media CTRL	Hymenoptaecin	2	Midgut	$1.497\times10^{5}\pm1.497\times10^{5}$	8	
N. ceranae only	Hymenoptaecin	2	Midgut	0±0	6	χ^2_4=7.5357, *P*=0.110
L. passim only	Hymenoptaecin	2	Midgut	$2.554\times10^{4}\pm2.554\times10^{4}$	8	
N. ceranae + *L. passim*	Hymenoptaecin	2	Midgut	$1.644\times10^{5}\pm5.880\times10^{4}$	7	
Sucrose CTRL	Hymenoptaecin	3	Midgut	$1.990\times10^{5}\pm1.109\times10^{5}$	8	
Media CTRL	Hymenoptaecin	3	Midgut	0±0	8	
N. ceranae only	Hymenoptaecin	3	Midgut	$6.179\times10^{5}\pm3.188\times10^{5}$	7	χ^2_4=6.1826, *P*=0.186
L. passim only	Hymenoptaecin	3	Midgut	$4.390\times10^{6}\pm4.279\times10^{6}$	7	
N. ceranae + *L. passim*	Hymenoptaecin	3	Midgut	$1.918\times10^{5}\pm8.571\times10^{4}$	8	
Sucrose CTRL	Hymenoptaecin	6	Midgut	$5.537\times10^{5}\pm2.004\times10^{5}$**ab**	7	
Media CTRL	Hymenoptaecin	6	Midgut	$4.815\times10^{4}\pm3.464\times10^{4}$**b**	8	
N. ceranae only	Hymenoptaecin	6	Midgut	$3.484\times10^{6}\pm3.258\times10^{6}$**a**	8	χ^2_4=12.825, *P*=0.012
L. passim only	Hymenoptaecin	6	Midgut	$1.638\times10^{5}\pm9.372\times10^{4}$**ab**	8	
N. ceranae + *L. passim*	Hymenoptaecin	6	Midgut	$3.501\times10^{5}\pm7.346\times10^{4}$**a**	8	
Sucrose CTRL	Hymenoptaecin	17	Midgut	$6.387\times10^{6}\pm5.654\times10^{6}$	7	
Media CTRL	Hymenoptaecin	17	Midgut	$2.183\times10^{7}\pm2.019\times10^{7}$	8	
N. ceranae only	Hymenoptaecin	17	Midgut	-‡	-	χ^2_3=0.65304, *P*=0.884
L. passim only	Hymenoptaecin	17	Midgut	$8.710\times10^{5}\pm4.265\times10^{5}$	8	
N. ceranae + *L. passim*	Hymenoptaecin	17	Midgut	$9.149\times10^{6}\pm6.075\times10^{6}$	8	

Supplementary Table 3.3 cont'd

Treatment	Gene	Day	Tissue type	Mean copies ± SE	n	Statistical comparison
Sucrose CTRL	Hymenoptaecin	1	Hindgut	$7.605\times10^{7}\pm5.587\times10^{7}$	5	
Media CTRL	Hymenoptaecin	1	Hindgut	$8.085\times10^{6}\pm1.808\times10^{6}$	7	
N. ceranae only	Hymenoptaecin	1	Hindgut	$3.986\times10^{7}\pm1.454\times10^{7}$	7	$F_{4,30}$=2.571, P=0.058
L. passim only	Hymenoptaecin	1	Hindgut	$2.277\times10^{9}\pm2.261\times10^{9}$	8	
N. ceranae + *L. passim*	Hymenoptaecin	1	Hindgut	$7.146\times10^{7}\pm3.871\times10^{7}$	8	
Sucrose CTRL	Hymenoptaecin	2	Hindgut	$7.547\times10^{7}\pm6.084\times10^{7}$	7	
Media CTRL	Hymenoptaecin	2	Hindgut	$5.286\times10^{6}\pm1.671\times10^{6}$	8	
N. ceranae only	Hymenoptaecin	2	Hindgut	$8.564\times10^{6}\pm4.726\times10^{6}$	6	$F_{4,30}$=0.374, P=0.826
L. passim only	Hymenoptaecin	2	Hindgut	$2.741\times10^{7}\pm1.636\times10^{7}$	8	
N. ceranae + *L. passim*	Hymenoptaecin	2	Hindgut	$1.105\times10^{7}\pm6.001\times10^{6}$	6	
Sucrose CTRL	Hymenoptaecin	3	Hindgut	$1.110\times10^{7}\pm7.193\times10^{6}$	8	
Media CTRL	Hymenoptaecin	3	Hindgut	$4.355\times10^{6}\pm1.040\times10^{6}$	8	
N. ceranae only	Hymenoptaecin	3	Hindgut	$6.358\times10^{6}\pm1.597\times10^{6}$	7	$F_{4,33}$=0.5461, P=0.703
L. passim only	Hymenoptaecin	3	Hindgut	$3.471\times10^{8}\pm3.274\times10^{8}$	7	
N. ceranae + *L. passim*	Hymenoptaecin	3	Hindgut	$1.010\times10^{7}\pm4.606\times10^{6}$	8	
Sucrose CTRL	Hymenoptaecin	6	Hindgut	$1.727\times10^{7}\pm8.037\times10^{6}$	8	
Media CTRL	Hymenoptaecin	6	Hindgut	$7.421\times10^{6}\pm1.438\times10^{6}$	8	
N. ceranae only	Hymenoptaecin	6	Hindgut	$4.316\times10^{7}\pm1.754\times10^{7}$	8	$F_{4,35}$=1.2179, P=0.321
L. passim only	Hymenoptaecin	6	Hindgut	$1.755\times10^{7}\pm3.990\times10^{6}$	8	
N. ceranae + *L. passim*	Hymenoptaecin	6	Hindgut	$9.437\times10^{6}\pm2.040\times10^{6}$	8	
Sucrose CTRL	Hymenoptaecin	17	Hindgut	$3.539\times10^{8}\pm3.084\times10^{8}$	7	
Media CTRL	Hymenoptaecin	17	Hindgut	$8.437\times10^{7}\pm5.655\times10^{7}$	8	
N. ceranae only	Hymenoptaecin	17	Hindgut	-‡	-	$F_{3,24}$=0.2313, P=0.874
L. passim only	Hymenoptaecin	17	Hindgut	$7.829\times10^{7}\pm3.243\times10^{7}$	6	
N. ceranae + *L. passim*	Hymenoptaecin	17	Hindgut	$3.888\times10^{8}\pm2.864\times10^{8}$	7	

Supplementary Table 3.3 cont'd

Treatment	Gene	Day	Tissue type	Mean copies ± SE	n	Statistical comparison
Sucrose CTRL	Defensin-1	1	Midgut	0±0**ab**	5	
Media CTRL	Defensin-1	1	Midgut	$1.708\times10^{6}\pm3.994\times10^{5}$**ab**	7	
N. ceranae only	Defensin-1	1	Midgut	$1.659\times10^{6}\pm8.169\times10^{5}$**a**	7	χ^2_4=10.419, *P*=0.034
L. passim only	Defensin-1	1	Midgut	$7.373\times10^{6}\pm6.524\times10^{6}$**ab**	7	
N. ceranae + *L. passim*	Defensin-1	1	Midgut	$8.647\times10^{5}\pm2.884\times10^{5}$**b**	8	
Sucrose CTRL	Defensin-1	2	Midgut	$5.387\times10^{5}\pm2.447\times10^{5}$	7	
Media CTRL	Defensin-1	2	Midgut	$8.918\times10^{5}\pm6.035\times10^{5}$	8	
N. ceranae only	Defensin-1	2	Midgut	$2.570\times10^{6}\pm1.476\times10^{6}$	5	χ^2_4=5.8909, *P*=0.207
L. passim only	Defensin-1	2	Midgut	$1.448\times10^{5}\pm6.069\times10^{4}$	8	
N. ceranae + *L. passim*	Defensin-1	2	Midgut	$8.928\times10^{6}\pm6.799\times10^{6}$	7	
Sucrose CTRL	Defensin-1	3	Midgut	$2.901\times10^{5}\pm2.814\times10^{5}$	8	
Media CTRL	Defensin-1	3	Midgut	$7.643\times10^{5}\pm3.252\times10^{5}$	8	
N. ceranae only	Defensin-1	3	Midgut	$3.413\times10^{6}\pm1.762\times10^{6}$	7	$F_{4,32}$=1.6197, *P*=0.193
L. passim only	Defensin-1	3	Midgut	$2.141\times10^{6}\pm1.526\times10^{6}$	7	
N. ceranae + *L. passim*	Defensin-1	3	Midgut	$1.995\times10^{6}\pm7.976\times10^{5}$	7	
Sucrose CTRL	Defensin-1	6	Midgut	$5.510\times10^{6}\pm2.676\times10^{6}$	7	
Media CTRL	Defensin-1	6	Midgut	$8.721\times10^{6}\pm5.803\times10^{6}$	8	
N. ceranae only	Defensin-1	6	Midgut	$5.743\times10^{6}\pm4.961\times10^{6}$	8	χ^2_4=3.4698, *P*=0.483
L. passim only	Defensin-1	6	Midgut	$2.328\times10^{6}\pm9.112\times10^{5}$	8	
N. ceranae + *L. passim*	Defensin-1	6	Midgut	$1.027\times10^{7}\pm5.365\times10^{6}$	8	
Sucrose CTRL	Defensin-1	17	Midgut	$4.306\times10^{6}\pm2.325\times10^{6}$	7	
Media CTRL	Defensin-1	17	Midgut	$8.321\times10^{7}\pm7.017\times10^{7}$	8	
N. ceranae only	Defensin-1	17	Midgut	-‡	-	χ^2_3=4.4904, *P*=0.213
L. passim only	Defensin-1	17	Midgut	$5.455\times10^{6}\pm2.572\times10^{6}$	8	
N. ceranae + *L. passim*	Defensin-1	17	Midgut	$4.313\times10^{6}\pm2.378\times10^{6}$	8	

Supplementary Table 3.3 cont'd

Treatment	Gene	Day	Tissue type	Mean copies ± SE	n	Statistical comparison
Sucrose CTRL	Defensin-1	1	Hindgut	$1.209\times10^{8}\pm2.182\times10^{7}$**ab**	5	
Media CTRL	Defensin-1	1	Hindgut	$1.156\times10^{8}\pm3.624\times10^{7}$**ab**	7	
N. ceranae only	Defensin-1	1	Hindgut	$8.441\times10^{7}\pm1.467\times10^{7}$**ab**	7	$F_{4,29}$=3.2226, P=0.026
L. passim only	Defensin-1	1	Hindgut	$1.143\times10^{8}\pm7.631\times10^{7}$**b**	7	
N. ceranae + *L. passim*	Defensin-1	1	Hindgut	$3.108\times10^{8}\pm1.252\times10^{7}$**a**	8	
Sucrose CTRL	Defensin-1	2	Hindgut	$2.998\times10^{8}\pm1.385\times10^{8}$	7	
Media CTRL	Defensin-1	2	Hindgut	$1.673\times10^{8}\pm4.189\times10^{7}$	8	
N. ceranae only	Defensin-1	2	Hindgut	$2.284\times10^{8}\pm9.558\times10^{7}$	6	$F_{4,30}$=0.3269, P=0.858
L. passim only	Defensin-1	2	Hindgut	$1.763\times10^{8}\pm8.254\times10^{7}$	8	
N. ceranae + *L. passim*	Defensin-1	2	Hindgut	$1.661\times10^{8}\pm4.708\times10^{7}$	6	
Sucrose CTRL	Defensin-1	3	Hindgut	$1.876\times10^{8}\pm5.355\times10^{7}$	8	
Media CTRL	Defensin-1	3	Hindgut	$2.201\times10^{8}\pm7.007\times10^{7}$	8	
N. ceranae only	Defensin-1	3	Hindgut	$1.425\times10^{8}\pm1.626\times10^{7}$	7	$F_{4,33}$=0.6436, P=0.635
L. passim only	Defensin-1	3	Hindgut	$1.022\times10^{9}\pm7.226\times10^{8}$	7	
N. ceranae + *L. passim*	Defensin-1	3	Hindgut	$1.997\times10^{8}\pm6.521\times10^{7}$	8	
Sucrose CTRL	Defensin-1	6	Hindgut	$2.014\times10^{8}\pm2.597\times10^{7}$	8	
Media CTRL	Defensin-1	6	Hindgut	$1.925\times10^{8}\pm2.549\times10^{7}$	8	
N. ceranae only	Defensin-1	6	Hindgut	$5.450\times10^{8}\pm3.042\times10^{8}$	8	$F_{4,35}$=0.8334, P=0.513
L. passim only	Defensin-1	6	Hindgut	$2.434\times10^{8}\pm4.293\times10^{7}$	8	
N. ceranae + *L. passim*	Defensin-1	6	Hindgut	$2.337\times10^{8}\pm6.291\times10^{7}$	8	
Sucrose CTRL	Defensin-1	17	Hindgut	$3.383\times10^{8}\pm1.909\times10^{8}$	7	
Media CTRL	Defensin-1	17	Hindgut	$2.834\times10^{8}\pm1.754\times10^{8}$	8	
N. ceranae only	Defensin-1	17	Hindgut	-‡	-	$F_{3,24}$=0.1352, P=0.938
L. passim only	Defensin-1	17	Hindgut	$1.554\times10^{8}\pm5.677\times10^{7}$	6	
N. ceranae + *L. passim*	Defensin-1	17	Hindgut	$5.988\times10^{8}\pm3.948\times10^{8}$	7	

Supplementary Table 3.3 cont'd

Treatment	Gene	Day	Tissue type	Mean copies ± SE	n	Statistical comparison
N. ceranae only	PTP3	1	Midgut	0 ± 0	7	W=35, *P*=0.204
N. ceranae + *L. passim*	PTP3	1	Midgut	$3.009 \times 10^5 \pm 2.580 \times 10^5$	8	
N. ceranae only	PTP3	2	Midgut	$5.803 \times 10^4 \pm 5.803 \times 10^4$	6	W=17.5, *P*=0.355
N. ceranae + *L. passim*	PTP3	2	Midgut	0 ± 0	7	
N. ceranae only	PTP3	3	Midgut	$9.199 \times 10^7 \pm 6.238 \times 10^7$	7	W=29, *P*=0.953
N. ceranae + *L. passim*	PTP3	3	Midgut	$8.987 \times 10^7 \pm 5.155 \times 10^7$	8	
N. ceranae only	PTP3	6	Midgut	$5.025 \times 10^9 \pm 1.775 \times 10^9$	7	t=0.5234, *P*=0.610
N. ceranae + *L. passim*	PTP3	6	Midgut	$6.731 \times 10^9 \pm 2.734 \times 10^9$	7	
N. ceranae only	PTP3	17	Midgut	-	-	-
N. ceranae + *L. passim*	PTP3	17	Midgut	$2.235 \times 10^9 \pm 1.037 \times 10^9$	5	
N. ceranae only	PTP3	1	Hindgut	0 ± 0	6	W=27, *P*=0.471
N. ceranae + *L. passim*	PTP3	1	Hindgut	$4.918 \times 10^4 \pm 4.918 \times 10^4$	8	
N. ceranae only	PTP3	2	Hindgut	$3.293 \times 10^5 \pm 2.490 \times 10^5$	6	W=15, *P*=0.599
N. ceranae + *L. passim*	PTP3	2	Hindgut	$1.480 \times 10^5 \pm 1.480 \times 10^5$	6	
N. ceranae only	PTP3	3	Hindgut	$3.093 \times 10^7 \pm 1.333 \times 10^7$	7	W=13, *P*=0.158
N. ceranae + *L. passim*	PTP3	3	Hindgut	$4.771 \times 10^6 \pm 3.607 \times 10^6$	7	
N. ceranae only	PTP3	6	Hindgut	$1.404 \times 10^8 \pm 4.226 \times 10^7$ **b**	8	W=51, *P*=0.05
N. ceranae + *L. passim*	PTP3	6	Hindgut	$5.445 \times 10^8 \pm 1.751 \times 10^8$ **a**	8	
N. ceranae only	PTP3	17	Hindgut	-‡	-	-
N. ceranae + *L. passim*	PTP3	17	Hindgut	$3.324 \times 10^8 \pm 1.150 \times 10^8$	6	

Supplementary Table 3.3 cont'd

Treatment	Gene	Day	Tissue type	Mean copies ± SE	n	Statistical comparison
L. passim only	LpLSU	1	Midgut	$1.046\times10^{8}\pm1.009\times10^{8}$**a**	7	W=48, *P*=0.007
N. ceranae + *L. passim*	LpLSU	1	Midgut	0±0**b**	8	
L. passim only	LpLSU	2	Midgut	$9.519\times10^{7}\pm8.241\times10^{7}$	7	W=32, *P*=0.360
N. ceranae + *L. passim*	LpLSU	2	Midgut	$8.925\times10^{6}\pm8.435\times10^{6}$	7	
L. passim only	LpLSU	3	Midgut	$2.966\times10^{7}\pm1.696\times10^{7}$**a**	7	W=52, *P*=0.002
N. ceranae + *L. passim*	LpLSU	3	Midgut	0±0**b**	8	
L. passim only	LpLSU	6	Midgut	$8.541\times10^{6}\pm7.257\times10^{6}$	7	W=24, *P*=0.648
N. ceranae + *L. passim*	LpLSU	6	Midgut	$2.119\times10^{7}\pm1.943\times10^{7}$	8	
L. passim only	LpLSU	17	Midgut	$2.070\times10^{6}\pm2.070\times10^{6}$	8	W=19, *P*=0.907
N. ceranae + *L. passim*	LpLSU	17	Midgut	$6.205\times10^{4}\pm6.205\times10^{4}$	5	
Media CTRL	LpLSU	1	Hindgut	$2.882\times10^{7}\pm1.949\times10^{7}$**b**	7	
L. passim only	LpLSU	1	Hindgut	$1.373\times10^{10}\pm3.373\times10^{9}$**a**	8	$F_{2,20}$=9.9489, *P*=0.001
N. ceranae + *L. passim*	LpLSU	1	Hindgut	$1.747\times10^{10}\pm7.902\times10^{9}$**a**	8	
Media CTRL	LpLSU	2	Hindgut	$5.144\times10^{6}\pm2.212\times10^{6}$**b**	7	
L. passim only	LpLSU	2	Hindgut	$2.540\times10^{10}\pm8.468\times10^{9}$**a**	8	$F_{2,18}$=9.4543, *P*=0.002
N. ceranae + *L. passim*	LpLSU	2	Hindgut	$3.140\times10^{10}\pm1.468\times10^{10}$**a**	6	
Media CTRL	LpLSU	3	Hindgut	$1.427\times10^{7}\pm5.843\times10^{6}$**b**	8	
L. passim only	LpLSU	3	Hindgut	$2.140\times10^{10}\pm3.755\times10^{9}$**a**	7	χ^2_2=12.615, *P*=0.002
N. ceranae + *L. passim*	LpLSU	3	Hindgut	$2.267\times10^{8}\pm1.194\times10^{8}$**b**	8	
Media CTRL	LpLSU	6	Hindgut	$4.079\times10^{6}\pm2.947\times10^{6}$**b**	8	
L. passim only	LpLSU	6	Hindgut	$1.036\times10^{10}\pm4.236\times10^{9}$**a**	8	$F_{2,21}$=4.4259, *P*=0.025
N. ceranae + *L. passim*	LpLSU	6	Hindgut	$1.367\times10^{10}\pm7.703\times10^{9}$**a**	8	
Media CTRL	LpLSU	17	Hindgut	$1.289\times10^{6}\pm5.063\times10^{5}$**b**	8	
L. passim only	LpLSU	17	Hindgut	$2.905\times10^{9}\pm2.756\times10^{9}$**a**	6	χ^2_2=13.867, *P*=0.001
N. ceranae + *L. passim*	LpLSU	17	Hindgut	$6.745\times10^{7}\pm2.549\times10^{7}$**a**	6	

[3]‡ indicates absence of samples due to bee death, presumably due to infection, before the sampling time point

[3]

Appendix C. Supplementary Information for Chapter 4

Supplementary Table 4.1. Endpoint, qPCR primers and standard curve information used in this study to verify *Nosema* spp., and quantify vitellogenin levels in collected bees

Assay	**Primer**	**Sequence**	**NT**	**Tm (°C)**	**Size**	**Melt temp (°C)**	**R^2**	**Efficiency (%)**	**Slope**	***Y*-int**	**Reference**
RpS5 endpoint and qPCR	RpS5 F	AATTATTTGGTCGCTGGAATTG	22	51.6	115	77.5-78	0.989	91.8	-3.535	41.855	Evans *et al.* 2006
	RpS5 R	TAACGTCCAGCAGAATGTGGTA	22	55.9							
N. ceranae endpoint	NoscRNAPol F2	TGGGTTCCCTAAACCTGGTGGTTT	24	64.0	662	-	-	-	-	-	Gisder *et al.* 2013
	NoscRNAPol R2	TCACATGACCTGGTGCTCCTTCT	23	63.5							
N. apis endpoint	NosaRNAPol F2	AGCAAGAGACGTTTCTGGTACCTCA	25	63.7	297	-	-	-	-	-	Gisder *et al.* 2013
	NosaRNAPol R2	CCTTCACGACCACCCATGGCA	21	65.0							
β-actin qPCR	Am-actin2-qF	CGTGCCGATAGTATTCTTG	19	58.0	271	87.5-88	0.990	91.4	-3.547	44.780	Mondet *et al.* 2014
	Am-Actin2-qR	CTTCGTCACCAACATAGG	18	57.6							
Vitellogenin qPCR	VgF	TCGACAACTGCGATCAAAGGA	21	56.6	164	82-83	0.985	92.3	-3.522	43.543	Schwarz *et al.* 2016
	VgR	TGGTCACCGACGATTGGATG	20	57.3							
L. passim cytb qPCR	Lpcytb_F2	AGTATGAGCAGTAGGTTTTATTATA	25	49.3	146	75.5	0.994	86	-3.699	37.973	Vejnovic *et al*. 2018
	Lpcytb_R	GCCAAACACCAATAACTGGTACT	23	55.4							

Supplementary Table 4.2. gBlock sequences used in this study to create standard curves, and determine vitellogenin levels in collected bees

Organism	Target	gBlock sequence	gBlock length (bp)
A. mellifera	RpS5	**TGAAATTGATTAAGGTATGTAAAATCCGGCAGTCACAGGTCG GAGCGCCACTAGTGTCATGGCCTTTTCCTGTTCACGCTTACTC CAAAGAAGACGTGAACGAAAGTTGTATGATAAATCATGGCTG AAATGGAAACATATGATGATATAGTGGTACCTACCACGACGAC ATTACCAGTGGCCCTTTCTGCAGAACTACCTGAAATTAAATTAT TTGGTCGCTGGAATTGTGATGATGTACAAGTGAATGATATGTC TTTACAAGATTATATTGCCGTTAAAGAGAAAAATGCAAAATAT TTACCACATTCTGCTGGACGTTATGCCGCAAAAAGATTTCGGA AAGCGCAATGTCCTATAGTCGAACGTTTGACAAACTCTTTAAT GATGCATGGTAGAAATAATGGGAAAAAGTTAATGGCAGTAA GAATTGTAAAACATGCCTTTGAAATAATTCACCTGCTCACGGG TGATAATCCTTTACAGGTTCTTGTGACTG**	499
A. mellifera	β-actin	**AGTGTTCGCAACTCGCGGCTCTCGAGTCTCGCTTCGTCGTGCC GATAGTATTCTTGCGGTGTCTCTTTGCCGATCAGCGATCGAGT ACTTTGTTGGTTACCTTCGATTCTAAAAGATAAACCAATAAGC CAACATGTCTGACGAAGAAGTTGCTGCACTCGTAGTTGACAA TGGCTCCGGTATGTGCAAAGCCGGTTTCGCCGGAGACGACGC ACCACGCGCCGTTTTCCCATCTATCGTCGGAAGACCACGCCAC CAGGGTGTCATGGTTGGCATGGGACAAAAGGATTCCTATGTT GGTGACGAAGCCCAATCAAAGAGAGGTATTCTTACCTTGAAA TACCCAATTGAGCATGGTATTGTTACCAACTGGGATGATATGG AGAAAATTTGGCATCACACTTTCTACAATGAACTTCGAGTGGC TCCCGAGGAACATCCGGTACTTCTCACTGAGGCACCTCTGAAT CCGAAGGCCAATCGTGAAAAGATGACACAA**	499

A. mellifera	Vitellogenin	**TTCAAAGCCATGGAGGACTCCGTGGGAGGGAAATGCGAGGT TCTCTACGATATAGCGCCTTTGTCGGACTTTGTGATCCACAGAT CGCCGGAATTGGTGCCGATGCCGACTTTGAAGGGCGATGGC CGCCACATGGAGGTGATCAAGATCAAGAACTTCGACAACTGC GATCAAAGGATAAATTATCATTTCGGTATGACCGACAACTCGA GGTTGGAACCTGGAACGAACAAGAATGGAAAGTTCTTCTCG AGATCTTCAACGAGTAGAATCGTTATCTCAGAAAGCCTGAAA CATTTCACCATCCAATCGTCGGTGACCACGAGCAAGATGATG GTCAGCCCTAGACTCTACGATCGTCAAAACGGATTGGTGCTTA GCAGAATGAACCTGACTTTAGCAAAGATGGAGAAAACGTCG AAACCTTTGCCTATGGTCGACAATCCAGAATCCACTGGCAATT TGGTTTACATCTACAATAATCCTTTCTCGGATGTCG**	499

Supplementary Table 4.3. Comparisons of ratios of total foragers by parasite treatment by day (with adjustment). Mixed spp. refers to bees inoculated with both *N. ceranae* and *L. passim*. Letters indicate statistically significant differences among treatments for a given time point (α=0.05; *p*=0.05)

Treatment	n (IB:BB)
Day 11	χ^2_3=3, *P*=0.392
Control	2:3
N. ceranae	2:3
L. passim	0:0
Mixed spp.	3:3
Day 15	$F_{3,4}$=1.59, *P*=0.325
Control	2:3
N. ceranae	0:0
L. passim	6:2
Mixed spp.	3:2
Day 16	χ^2_3=2.62, *P*=0.453
Control	5:6
N. ceranae	6:4
L. passim	9:4
Mixed spp.	13:6
Day 17	$F_{3,4}$=2.27, *P*=0.223
Control	12:10
N. ceranae	13:11
L. passim	6:6
Mixed spp.	30:7
Day 18	χ^2_3=1.1405, *P*=0.767
Control	25:27
N. ceranae	17:14
L. passim	7:5
Mixed spp.	42:25
Day 19	χ^2_3=91.2, *P*<0.001
Control	30:31 *c*
N. ceranae	20:18 *c*
L. passim	20:13 *b*
Mixed spp.	55:19 *a*
Day 20	χ^2_3=12.6, *P*<0.001
Control	31:32 *b*
N. ceranae	27:17 *ab*
L. passim	30:26 *ab*
Mixed spp.	53:30 *a*
Day 21	$F_{3,8}$=0.75, *P*=0.551
Control	34:30
N. ceranae	31:25
L. passim	22:17
Mixed spp.	47:30

Supplementary Table 4.4. Comparisons of ratios of pollen foragers by parasite treatment by day (with adjustment). Mixed spp. refers to bees inoculated with both *N. ceranae* and *L. passim*. Letters indicate statistically significant differences among treatments for a given time point (α=0.05; *p*=0.05)

Treatment	n (IB:BB)
Day 15	χ^2_3=3, *P*=0.392
Control	0:0
N. ceranae	0:0
L. passim	5:1
Mixed spp.	9:2
Day 16	χ^2_3=0.18, *P*=0.98
Control	1:2
N. ceranae	2:1
L. passim	6:2
Mixed spp.	7:1
Day 17	χ^2_3=11.4, *P*<0.001
Control	6:6 *b*
N. ceranae	6:7 *b*
L. passim	4:3 *ab*
Mixed spp.	23:3 *a*
Day 18	χ^2_3=11.1, *P*<0.001
Control	17:18 *b*
N. ceranae	6:11 *b*
L. passim	0:0 *b*
Mixed spp.	32:14 *a*
Day 19	χ^2_3=3.06, *P*=0.383
Control	19:18
N. ceranae	8:7
L. passim	9:6
Mixed spp.	33:11
Day 20	$F_{3,8}$=3.08, *P*=0.094
Control	19:18
N. ceranae	12:9
L. passim	11:10
Mixed spp.	28:13
Day 21	$F_{3,8}$=0.752, *P*=0.551
Control	17:21
N. ceranae	4:8
L. passim	13:6
Mixed spp.	25:19

Supplementary Table 4.5. Comparisons of ratios of nectar foragers by parasite treatment by day (with adjustment). Mixed spp. refers to bees inoculated with both *N. ceranae* and *L. passim*. Letters indicate statistically significant differences among treatments for a given time point (α=0.05; *p*=0.05)

Treatment	n (IB:BB)
Day 11	χ^2_3=3.000, *P*=0.392
Control	1:2
N. ceranae	1:2
L. passim	0:0
Mixed spp.	2:2
Day 15	χ^2_3=3.000, *P*=0.392
Control	1:2
N. ceranae	0:0
L. passim	0:0
Mixed spp.	0:0
Day 16	χ^2_3=0.43, *P*=0.934
Control	3:3
N. ceranae	2:1
L. passim	2:1
Mixed spp.	5:4
Day 17	χ^2_3=4.945, *P*=0.176
Control	6:4
N. ceranae	6:3
L. passim	1:2
Mixed spp.	7:4
Day 18	$F_{3,4}$=0.659, *P*=0.619
Control	7:8
N. ceranae	10:2
L. passim	5:3
Mixed spp.	17:5
Day 19	χ^2_3=12.3, *P*<0.001
Control	11:13 *b*
Nosema ceranae	12:11 *b*
Lotmaria passim	10:6 *b*
Mixed spp.	22:8 *a*
Day 20	$F_{3,8}$=1.42, *P*=0.306
Control	11:13
N. ceranae	15:8
L. passim	19:16
Mixed spp.	25:17
Day 21	$F_{3,8}$=2.25, *P*=0.160
Control	17:9
N. ceranae	25:15
L. passim	8:10
Mixed spp.	21:10

Supplementary Table 4.6. Ratio comparisons of average forager age (days old) by parasite treatment across all three replicates. Mixed spp. refers to bees inoculated with both *N. ceranae* and *L. passim*. Letters indicate statistically significant differences among treatments for a given time point (α=0.05; *p*=0.05)

Treatment	IB mean age± SE:BB mean age ± SE	Statistical comparison	n (IB:BB)
Control	19.34 ± 0.13:19.15 ± 0.15	χ^2_1=1.26, *P*=0.263	111:110
Nosema ceranae	19.41 ± 0.16:19.28 ± 0.23	χ^2_1=0.20, *P*=0.655	84:60
Lotmaria passim	19.26 ± 0.21:19.90 ± 0.16	χ^2_1=2.59, *P*=0.11	68:41
Mixed spp.	18.86 ± 0.11:19.45 ± 0.18	χ^2_1=7.03, *P*<0.001	225:82

Supplementary Table 4.7. Comparisons of ratios of vg expression by parasite treatment at first instance of foraging (FI), and 7 and 14 days of age for paint-marked NEBs. Mixed spp. refers to bees inoculated with both *N. ceranae* and *L. passim*. α=0.05; *p*=0.05

Treatment	n (IB:BB)
D7 IB:BB	$F_{3,8}$=0.689 *P*=0.584
Control	12:12
Nosema ceranae	12:12
Lotmaria passim	12:12
Mixed spp.	12:12
D14 IB:BB	$F_{3,8}$=5.802 *P*=0.122
Control	12:12
Nosema ceranae	12:12
Lotmaria passim	12:12
Mixed spp.	12:12
FI IB:BB	$F_{3,8}$=3.155 *P*=0.086
Control	12:12
Nosema ceranae	12:12
Lotmaria passim	12:12
Mixed spp.	12:12

Supplementary Table 4.8. Comparisons of vg expression (copies / bee) by parasite treatment and inoculation status (IB or BB) at first instance of foraging for paint-marked NEBs across all three replicates. Mixed spp. refers to bees inoculated with both *N. ceranae* and *L. passim*. α=0.05; *p*=0.05

Treatment	IB mean vg expression (copies/bee)± SE:BB mean vg expression (copies/bee) ± SE	Statistical comparison	n (IB:BB)
Control	$3.94\times10^9 \pm 1.87\times10^9$:$2.12\times10^9 \pm 07.44\times10^8$	χ^2_1=1.09, *P*=0.296	12:12
Nosema ceranae	$1.16\times10^9 \pm 6.41\times10^8$:$4.56\times10^9 \pm 6.41\times10^8$	χ^2_1=3.82, *P*=0.051	12:12
Lotmaria passim	$2.26\times10^9 \pm 1.40\times10^9$:$2.91\times10^9 \pm 1.07\times10^9$	χ^2_1=6.56, *P*=0.011	12:12
Mixed spp.	$4.97\times10^8 \pm 1.06\times10^8$:$4.00\times10^9 \pm 1.53\times10^9$	χ^2_1=38.9, *P*<0.001	12:12

Supplementary Table 4.9. Comparisons of vg expression by parasite treatment and foraging status foraging (foraging [F] vs. not foraging [NF]) at first instance of foraging paint-marked NEBs across all three replicates. Mixed spp. refers to bees inoculated with both *N. ceranae* and *L. passim*. α=0.05; *p*=0.05

Treatment	F mean vg expression (copies/bee)± SE:NF mean vg expression (copies/bee) ± SE	Statistical comparison	n (F:NF)	n (IB:BB)
Control	$1.44\times10^9 \pm 4.36\times10^9$:$8.53\times10^9 \pm 3.74\times10^9$	χ^2_1=7.39, *P*<0.001	19:5	12:12
Nosema ceranae	$1.70\times10^9 \pm 6.83\times10^8$:$8.47\times10^9 \pm 1.73\times10^9$	$F_{1,22}$=36.6, *P*<0.001	18:6	12:12
Lotmaria passim	$6.12\times10^8 \pm 2.19\times10^8$:$6.54\times10^9 \pm 1.95\times10^9$	χ^2_1=25.7, *P*<0.001	16:8	12:12
Mixed spp.	$1.49\times10^9 \pm 6.96\times10^8$:$6.87\times10^9 \pm 2.19\times10^9$	χ^2_1=14.8, *P*<0.001	16:8	12:12

Supplementary Table 4.10. Ratio comparisons of weights (mg) of pollen and nectar loads by parasite treatment (with adjustment) on day 21 (last day of experiment) across all three replicates. Mixed spp. refers to bees inoculated with both *N. ceranae* and *L. passim*. Letters indicate statistically significant differences among treatments for a given time point (α=0.05; *p*=0.05)

Treatment	IB mg ± SE:BB mg ± SE	n(IB:BB)
Pollen	$F_{3,4}$=0.924, *P*=0.472	
Control	7.89 ± 1.37:7.34 ± 0.80	20:27
Nosema ceranae	4.35 ± 1.52:5.41 ± 1.00	11:15
Lotmaria passim	7.91 ± 1.55:6.86 ± 1.30	17:12
Mixed spp.	11.01 ± 1.40:9.57 ± 1.62	25:22
Nectar	χ^2_3=0.1008, *P*=0.992	
Control	8.56 ± 1.15:9.12 ± 1.78	14:10
Nosema ceranae	7.18 ± 1.61:7.74 ± 2.49	17:16
Lotmaria passim	6.32 ± 2.85:3.90 ± 0.90	9:12
Mixed spp.	8.98 ± 2.67:14.87 ± 4.31	10:12