MULTITROPHIC INTERACTIONS AND THE EXOTIC LIGHT BROWN APPLE MOTH, *EPIPHYAS POSTVITTANA*, IN CALIFORNIA

by
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ABSTRACT

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Invasive species are recognized as one of the main drivers of global environmental change. The majority of invasive species escape from their coevolved natural enemies (predators, parasitoids and parasites), but in some cases can either act as a transport vector for coevolved parasites, or encounter biotic resistance from resident competitors or enemies, in a new region. When resident enemies are unable to suppress an invasive species, specialist natural enemies from the native range can be imported and implemented to reduce its abundance in a novel environment (classical biological control). As top-down effects of natural enemies can interact with bottom-up effects from host plants, it is of increasing interest to understand how multitrophic interactions influence the suppression of invasive species. The light brown apple moth, *Epiphyas postvittana* (Walker) (Lepidoptera: Tortricidae), provides a unique opportunity to examine mechanisms for the suppression of an exotic species and in the context of novel multitrophic interactions. Native to Australia, *E. postvittana* is a highly polyphagous leafroller that was confirmed to be present in coastal California in 2006. For my dissertation research I have focused on several different aspects of the trophic interactions among *E. postvittana* and its natural enemies.

Firstly, I investigated the occurrence and fitness consequences of infanticide for *Goniozus jacintae* Farrugia, a coevolved gregarious larval ectoparasitoid of *E. postvittana*. Bethylid parasitoids, such as *G. jacintae*, have long been recognized for their aggressive adult behavior and their use of infanticide to kill the offspring of competing females. In this laboratory study I investigated the clutch size and handling time of *G. jacintae*, compared its life history performance between primary and secondary (laid after infanticide events) broods, and estimated lipid and protein concentrations in the hemolymph of non-parasitized and parasitized hosts. I found that secondary clutches were significantly larger than primary clutches in ovicide treatments and also experienced greater brood survivorship. Lipid concentrations were consistently higher in the hemolymph of parasitized hosts and protein concentrations were also higher until egg hatch when parasitoid larvae began to consume the resources available. This study was the first to provide evidence that improved nutritional quality could be an important benefit of infanticide for an insect parasitoid, allowing for larger clutch size and improved brood survivorship among secondary broods.

Subsequently, I discovered a microsporidian pathogen infecting field populations of *E. postvittana* in California and I investigated both its identity and pathogenicity. Using

ultrastructure of different spore stages in the life cycle and phylogenetic analysis of sequences from selected genetic markers (ITS, SSU and RPB1), I was able to confirm that the microsporidian was a member of the *Nosema fumiferanae* species complex (*N. fumiferanae postvittana* subsp. n.). I was also able to confirm that this microsporidian can be vertically transmitted and that it has significant negative effects on the life-history performance of *E. postvittana* under laboratory conditions. To further explore the potential of this microsporidian infection to provide biotic resistance to the invasiveness of *E. postvittana*, I further examined its pathogenicity in the context of dose-response relationships and the latent period of infection in the laboratory, and quantified pathogen prevalence and intensity in the field using quantitative real-time PCR (qPCR) for five populations in the San Francisco Bay Area of California. In the laboratory, the median lethal dose (LD_{50}) was estimated to be 1.8×10^4 spores, the mean latent period for infection with 10^3 spores was 12.67 days, and compared to healthy larvae, those infected with up to 10^5 spores showed a reduction in intrinsic rate of natural increase from 0.18 to 0.008. From the field sampling I detected *N. fumiferanae postvittana* in all five populations with an overall prevalence of 5% and a mean microsporidian intensity of 226 spores. Although the laboratory results demonstrated the potential for host suppression, the field sampling indicated that the prevalence and intensity were too low to account for the continued decline in population densities of *E. postvittana* in coastal California.

Finally, I investigated the role of multitrophic interactions among *E. postvittana*, its host plants, and its resident enemies in California. In a common garden experiment in the field, parasitism rates of egg masses and larvae by resident parasitoids in the field were found to vary among host plant species, with a higher probability of egg parasitism on taller plants and a higher probability of larval parasitism on shorter plants. In the laboratory, parasitoid search time for an egg mass varied among plant species, but longer search times did not necessarily correspond to lower rates of egg parasitism in the field. When controlling for plant species, the probability of a parasitoid contacting an egg mass decreased with increasing trichome density. I also found significant effects of plant diet on the fitness of both healthy and microsporidian-infected *E. postvittana* larvae under laboratory conditions, with evidence of synergistic effects between diet and infection for some host plant species. Overall, I demonstrated significant variation in the extent of enemy-free space for *E. postvittana* based on plant species, but not plant origin.

My dissertation highlights the importance of biotic resistance in buffering a resident community against an exotic invader and demonstrates that this resistance is often dependent on complex multitrophic interactions.

Dedication

To my parents, Anita and James Hopper, and my fiancé, Gerid Ollison, for their love, support, and inspiration, and pushing me to be my best in all realms of life.

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INTRODUCTION

Biotic invasions are recognized as being one of the main drivers of global environmental change (Sala et al., 2000; Tylianakis et al., 2008). Invasive species can disrupt and modify invaded communities through direct and indirect interactions and cause both ecological and economic repercussions (Mack et al., 2000; Ricciardi et al., 2013). The success of an invasive species is dependent on a variety of abiotic and biotic factors, such as climate compatibility, propagule pressure, resource abundance, adaptive traits, and the absence of resident competitors and natural enemies (Strayer et al., 2006; Davis, 2009; Simberloff, 2009; Ricciardi et al., 2013). The majority of invasive species escape from their coevolved natural enemies (Keane and Crawley, 2002; Mitchell and Power, 2003; Torchin et al., 2003), but in some cases can either act as a transport vector for coevolved parasites (Strauss et al., 2012), or encounter biotic resistance from novel resident competitors or enemies in a new region (Elton, 1958; Maron and Vila, 2001; Levine et al., 2004; Carlsson et al., 2011; Dumont et al., 2011; Kimbro et al., 2013; Alofs and Jackson, 2014). In the case of enemy release, a potential approach to reduce the impact of an exotic species is classical biological control, through importation of specialist natural enemies from the region of origin into the novel range to suppress the invasive population (Van Driesche et al., 2008). As top-down effects from enemies do not occur in isolation, it is essential to understand the outcomes of multitrophic interactions among resident species and novel invasive species (Harvey et al., 2010; Chabaane et al., 2015) to implement the best management strategies.

The light brown apple moth, *Epiphyas postvittana* (Walker) (Lepidoptera: Tortricidae), provides a unique opportunity to examine multitrophic interactions and potential mechanisms for the suppression of an exotic generalist herbivore. Native to Australia with a host plant range of over 500 plant species, *E. postvittana* was confirmed to be present in California in 2006 (Brown, 2007) and initially increased in abundance at an exponential rate in four counties that were monitored extensively from 2007 to 2010 (Suckling et al., 2014). As a new invader, the economic impact of *E. postvittana* in the USA was initially predicted to be $86-150 million annually from crop damage, preventative treatments, and trade restrictions (Fowler et al., 2009). Consequently, the United States Department of Agriculture initiated an eradication program. Control measures for this exotic moth on a global scale have included the use of insecticides, biopesticides, pheromone mating disruption, sterilized insect technology, post-harvest treatments, and biological control (Suckling and Brockerhoff, 2010). Biological control agents from Australia have included several parasitoids such as the solitary endoparasitoid *Dolichogenidea tasmanica* (Cameron) and the gregarious larval ectoparasitoid, *Goniozus jacintae* Farrugia (Suckling and Brockerhoff, 2010).

To better understand the biology of *G. jacintae* as a potential classical biological control agent, I investigate the occurrence and fitness consequences of infanticide in Chapter 1. Bethylid parasitoids, such as *G. jacintae*, have long been recognized for their aggressive competitive interactions (Hardy and Blackburn, 1991) and their use of infanticide to kill the offspring of competitors (Mayhew, 1997). Females of *G. jacintae* host feed on individuals selected for oviposition, temporarily paralyze them with venom, and partially guard their brood. I compare the life history performance of parasitoid offspring from primary clutches with those from secondary clutches laid after infanticide events. I also compare the concentration of proteins and

lipids in the hemolymph of non-parasitized and parasitized hosts to evaluate the possible nutritional benefits of infanticide for *G. jacintae*.

Following this study, it became clear that classical biological control would not be needed for *E. postvittana* in California as populations in Santa Cruz and San Francisco, the focal points of establishment, were observed to be in decline (Bürgi et al., 2015). After its accidental introduction to California, *E. postvittana* attracted the attention of a wide range of resident parasitoids (Wang et al., 2012; Bürgi and Mills, 2014; Bürgi et al., 2015) and generalist predators (Hogg et al., 2013) that contributed a significant level of biotic resistance. While many of the exotic species that establish in new geographic regions fail to become truly invasive, the extent to which biotic resistance may be responsible remains largely unknown as such populations are both more difficult to detect and to study (Zenni and Nunez, 2013). In other cases, however, exotic species exhibit an initial phase of population growth and spread, followed by a subsequent phase of natural decline (Simberloff and Gibbons, 2004; Zenni and Nunez, 2013). Such species, including *E. postvittana*, provide great opportunities to study the factors that limit the potential of invasive species (Zenni and Nunez, 2013).

Bürgi et al. (2015) investigated the effect of resident parasitoids on the dynamics of *E. postvittana* populations in San Francisco and Santa Cruz over a four-year period. Although they documented substantial levels of parasitism, they found no evidence for an effect on the per capita population growth rates of this newly adopted host. In contrast, they found evidence of strong compensatory feedback [a decrease in per capita growth rate with increasing population size (Herrando-Perez et al., 2012)] due to unknown factors. While factors contributing to population declines of naturalized exotic species are often poorly understood, pathogens have occasionally been considered to be at least partially responsible (Simberloff and Gibbons, 2004). The influence of pathogens on insect populations has often been overlooked in comparison to that of parasitoids and predators, as they are more difficult to detect and their effects are not as easily quantified (Anderson and May, 1981; Solter, 2014).

During the course of my dissertation, I discovered an infection by a microsporidium resembling a *Nosema* species in a laboratory colony and in field populations of *E. postvittana*. Microsporidia are a diverse group of intracellular pathogens related to the fungi (Hirt et al., 1999; Van de Peer et al., 2000; Fischer and Palmer, 2005; Karpov et al., 2014; Keeling, 2014) and comprise over 185 described genera and over 1300 species that are found in protists, invertebrates and vertebrates, including humans (Solter et al., 2012a; Vavra and Lukes, 2013). There are more than 150 described species in the genus *Nosema* (Microsporidia: Nosematidae) that are associated with 12 different orders of insects (Becnel and Andreadis, 2014). The type species of the genus, *Nosema bombycis* (Nägeli) was the first member of the Microsporidia to be described, and is the causal agent of pébrine disease in the silkworm, *Bombyx mori* L. (Vavra and Lukes, 2013; Becnel and Andreadis, 2014). In addition to the impact of *N. bombycis* in sericulture, *N. apis* Zander and *N. ceranae* Fries cause nosemosis in honeybees, *Apis mellifera* L. and *A. cerana* F., and have been suggested to be among the possible factors leading to their decline (Paxton et al., 2007). Several microsporidian species have also expanded their geographic range either as accidental or deliberate introductions. *N. ceranae,* suggested to originate from *A. cerana* in Asia (Fries et al., 1996; Gomez-Moracho et al., 2015), has been found in Europe, North America, and South America where it infects *A. mellifera* and bumblebees (Graystock et al., 2013). *N. tyriae*

Canning was accidentally introduced from Europe to North America along with the deliberate introduction of its host, the cinnabar moth, *Tyria jacobaeae* (L.) (Hawkes, 1973), and *N. lymantriae* Weiser and *Vairimorpha disparis* (Timofejeva) were deliberately introduced from Europe to North America in trial studies for biological control of the gypsy moth, *Lymantria dispar* (L.) (Hajek and Delalibera, 2010).

In Chapter 2, I characterize the pathogenicity, transmission pathways, and morphology of this *Nosema* isolate from *E. postvittana* (hereafter *N. fumiferanae postvittana*). Three commonly used genetic markers (ITS, SSU, and RPB1) are sequenced to identify the isolate and compare it to other *Nosema* species from lepidopteran hosts. I include further TEM imaging of *Nosema fumiferanae* from *C. fumiferana* (hereafter *N. fumiferanae*) as the previous ultrastructural images did not include the mature spore stage (Percy, 1973). I compare the ultrastructure of the two *N. fumiferanae* isolates, and relate the sequence and ultrastructure characteristics to other microsporidia in the *N. bombycis* group.

To understand the significance of the novel pathogen-host relationship between *N. fumiferanae postvittana* and *E. postvittana*, in Chapter 3, I conduct additional laboratory experiments and field sampling. The objectives of the laboratory experiments are to 1) estimate the median lethal dose (LD_{50}) and the effect of dose ingested on the life-history performance of *E. postvittana* larvae as key aspects of the pathogenicity of *N. fumiferanae postvittana*, and 2) estimate the latent period (time from initial host infection to production of infectious spores), an essential parameter for understanding disease dynamics (Anderson and May, 1981; Briggs and Godfray, 1995; Goertz and Hoch, 2011; Solter, 2014). The objective of the field sampling is to determine the prevalence (percentage of hosts infected at a particular point in time) and intensity (spore load of an infected host at a particular point in time) of infection (Bush et al., 1997) by *N. fumiferanae postvittana* in five field populations of *E. postvittana* in coastal California using quantitative real-time PCR (qPCR). I use qPCR as a tool for pathogen detection and quantification as low intensity infections are easily overlooked through light microscopy (Refardt and Ebert, 2006).

As *E. postvittana*, an exotic generalist herbivore, feeds on plants from a wide range of families, diet may have an important influence on its performance and potential for enemy-free space with respect to resident parasitoids and pathogens. Multitrophic interactions among native plants, insect herbivores and their enemies (predators, parasitoids and pathogens) have been studied extensively, primarily from a perspective of the bottom-up effects of plant quality and the top-down effects of enemies on the performance and diet breadth of herbivores (Price et al., 1980; Schoonhoven et al., 2005; Singer and Stireman, 2005; Mooney et al., 2012). Such studies have generated three key hypotheses regarding herbivore performance; that host plant specialists are better adapted to using their host plants than generalists, and consequently that specialists experience more enemy-free space than generalists on shared host plants, while generalists experience increased exposure to natural enemies when feeding on low quality plants (Mooney et al., 2012). Multitrophic interactions can be influenced by abiotic factors and a variety of plant traits including architecture, morphological adaptations, nutritional quality, and the direct and indirect effects of chemical defenses (Gols, 2014; Reinecke and Hilker, 2014).

Both plant architecture (Andow and Prokrym, 1990) and plant volatiles (Dicke and Baldwin,

2010; Pierik et al., 2014) can influence the foraging success of herbivores and their natural enemies (Gols, 2014). In addition, secondary plant metabolites and plant nutritional quality can also affect the growth and survivorship of herbivores, and consequently their suitability for the growth, survival and reproduction of their natural enemies (Gols, 2014). On the one hand, secondary plant metabolites can have a positive influence on natural enemies by stressing insect herbivores and either increasing the risk of enemy attack (Benrey and Denno, 1997), or decreasing resistance to enemy attack (Cory and Hoover, 2006; Bukovinszky et al., 2009; Gassmann et al., 2010). On the other hand, these metabolites can also have a negative influence on natural enemies through increased resistance of herbivores to enemy attack (Singer et al., 2009; Gassmann et al., 2010), and/or reduced transmission rates in the case of pathogens (Richardson et al., 2015). Thus the direct and indirect effects of plant traits are not independent and may interact in influencing multitrophic interactions (Gols, 2014). For example, there is often a trade-off between the nutritional quality of a plant for an herbivore and the extent of enemy-free space that it provides (Price et al., 1980; Singer et al., 2004; Gassmann et al., 2010).

In Chapter 4, I use both field and laboratory experiments to determine the influence of host plants on parasitism and infection of *E. postvittana*. To examine host plant effects on parasitism of egg masses and larvae in the field I use a common garden experiment with both native and exotic plant species in a heterogeneous urban landscape. In addition, in the laboratory, I monitor the foraging of *Trichogramma fasciatum* (Perkins) for host egg masses on excised leaves to investigate the consequences of leaf characteristics for parasitoid foraging success. To determine the effect of host plant quality on the performance of *E. postvittana*, I record their growth, survival and reproduction when fed on a variety of different diets under laboratory conditions. Finally, to examine whether diets differ in their potential to provide protection from natural enemies, I compare the effect of diet on healthy *E. postvittana* larvae and those infected with the microsporidian pathogen.

CHAPTER 1

CONSEQUENCES OF INFANTICIDE FOR A GREGARIOUS ECTOPARASITOID OF LEAFROLLER LARVAE

1.1 ABSTRACT

In this laboratory study we investigated the clutch size and handling time of the ectoparasitic wasp, *Goniozus jacintae*, compared its life history performance between primary and secondary (laid after infanticide events) broods, and estimated lipid and protein concentrations in the hemolymph of non-parasitized and parasitized hosts. We found that *G. jacintae* temporarily paralyzed its host larvae, *Epiphyas postvittana*, for 66 min and briefly guarded its brood for 66 min. Clutch size of *G. jacintae* increased from two to seven with increasing larval fresh weight of its host, and both ovicide and larvicide of primary clutches occurred in 81% of encounters. Secondary clutches of *G. jacintae* were significantly larger than primary clutches in two of three ovicide treatments for the same host individuals. Secondary clutches also experienced greater brood survivorship than primary clutches. Lipid concentrations were consistently higher in the hemolymph of parasitized hosts and protein concentrations were initially higher (until egg hatch), but increased at a lower rate in parasitized hosts compared to non-parasitized hosts. This study is the first to provide evidence that improved nutritional quality could be an important benefit of infanticide for an insect parasitoid, allowing for larger clutch size and improved brood survivorship among secondary broods.

1.2 INTRODUCTION

Exploitative competition within the broods of gregarious parasitoids has often been minimized through the evolution of exact clutch sizes allowing parent females to accurately assess the quality of a host and adapt their clutch size to match the availability of resources for offspring development. Clutch size in gregarious parasitoids is also known to be influenced by other factors such as host density, host quality, energetic reserves, and presence of competitors (Godfray, 1994). For example, superparasitism by gregarious parasitoids frequently results in the oviposition of a smaller secondary clutch, and in most cases the resultant offspring develop to become smaller adults as a consequence of resource sharing (Godfray, 1994; Visser and Rosenheim, 1998). In some cases, however, the size of a secondary clutch may not be greatly reduced in the presence of competitors if the parent female has the ability to successfully secure the host resources through infanticide of the primary clutch.

Infanticide occurs when a parent female parasitoid either removes or kills the eggs or larvae of competitors to eliminate or at least reduce competition for the remaining host resources for its own offspring (van Alphen and Visser, 1990). Infanticide is known to occur among parasitoids in the Aphelinidae (Netting and Hunter, 2000), Bethylidae, Braconidae, and Pteromalidae (Godfray, 1994; Mayhew, 1997), Dryinidae (Yamada and Kitashiro, 2002; Ito and Yamada, 2005), Encyrtidae (Tena et al., 2008), Eupelmidae (Rojas-Rousse et al., 2005), and Ichneumonidae (Takasuka and Matsumoto, 2011). In addition to infanticide, parasitoids can respond to previously parasitized, but otherwise suitable, hosts by rejecting them and searching for a non-parasitized host (Mayhew, 1997), by host feeding on their hemolymph to gain additional resources for search and/or egg production (Tena et al., 2008), or by superparasitizing (intraspecific) or multiparasitizing (interspecific) them by laying additional eggs (van Alphen and Visser, 1990).

Based on an evolutionary model of infanticide, Strand and Godfray (1989) suggested that ovicide is more likely to occur in parasitoids when: (1) the first clutch has a competitive advantage for superparasitized hosts; (2) non-parasitized hosts are rare; (3) time taken to perform ovicide is minimal; and (4) the risk of removing an individual's own clutch is low. In support of their model, Strand and Godfray (1989) found that ovicide by the gregarious ectoparasitoid, *Bracon hebetor*, occurred more frequently when either the time to encounter unparasitized hosts or the frequency of encountering previously parasitized hosts increased. In support of this evolutionary model, experiments have shown that ovicide is more frequent when the first clutch has a competitive advantage, and when the time taken to perform ovicide was minimal (Mayhew, 1997; Netting and Hunter, 2000; Yamada and Kitashiro, 2002). Although Tena et al. (2008) found ovicide to be more likely when the first clutch had a competitive advantage, there was a substantial increase in the handling time (time from first encounter to departure from a host) required for secondary clutches on previously parasitized hosts compared to primary clutches on non-parasitized hosts.

The fitness consequences of infanticide for a secondary clutch can be a reduction in offspring size, as found by (Mayhew, 1997) for the gregarious ectoparasitoid, *Laelius pedatus* (Say). For example, a previously parasitized host may be a lower quality host for parasitoid offspring compared to a non-parasitized host if it deteriorates post parasitism (Strand and Godfray, 1989;

Mayhew, 1997). Alternatively, a previously parasitized host could be a better quality resource than a non-parasitized host if the host encapsulation response is completely or partially suppressed (van Alphen and Visser, 1990; Tena et al., 2008) or if the nutritional quality is increased (Harvey et al., 2013). Host nutritional quality can be increased if injection of parasitoid venom (Asgari and Rivers, 2011) elevates lipid levels in the hemolymph (e.g.,Nakamatsu and Tanaka, 2004), if parasitic castration of the host occurs, as this has been linked to an increase in protein and triacylglycerol concentration in the hemolymph (Pennacchio et al., 1995), if polydnaviruses initially increase trehalose levels in the hemolymph (e.g. Nakamatsu et al., 2001), or if polydnaviruses or symbionts redirect the allocation of metabolic resources from host metabolism and reproduction to juvenile parasitoid growth (Rahbe et al., 2002; Thompson et al., 2005).

In this study we investigate the occurrence and fitness consequences of parasitism and infanticide for *Goniozus jacintae* Farrugia, a gregarious larval ectoparasitoid of the light brown apple moth, *Epiphyas postvittana* (Walker) (Fig. S1.1). Bethylid parasitoids, such as *G. jacintae*, have long been recognized for their aggressive interspecific and intraspecific competitive interactions (Hardy and Blackburn, 1991) and their use of ovicide and/or larvicide (Mayhew, 1997). Larvae of *E. postvittana* are susceptible to parasitism by *G. jacintae* from the 3rd to the 5th instar and also during the extended 6th instar, but parasitism primarily occurs during the 4th and 5th instars (Danthanarayana, 1980). Females of *G. jacintae* host feed on individuals selected for oviposition, temporarily paralyze them with venom, and partially guard their brood. We compare the life history performance of parasitoid offspring from primary clutches with those from secondary clutches laid after infanticide events. We also compare the concentration of proteins and lipids in the hemolymph of non-parasitized and parasitized hosts to evaluate the potential nutritional benefits of infanticide for *G. jacintae*.

In general, clutch size for gregarious ectoparasitoids increases with host size (Godfray, 1994; Harvey, 2005), as has been documented for the congeneric parasitoid, *G. nephantidis* (Muesebeck) (Hardy et al., 1992). In this context, we hypothesize that *G. jacintae* females should match their clutch size to host size for both primary and secondary clutches. We also hypothesize that the size of a secondary clutch should be greater than or equal to that of a primary clutch for cases of ovicide in which the eggs of the primary clutch had not yet hatched into larvae. As eggs of most ectoparasitoids do not increase in size before larval hatch, no host resources are used, and thus host quality should remain the same for secondary clutches as it was for primary clutches (Goubault et al., 2007). Moreover, if previous parasitism does increase the nutritional quality of a host through the action of venom, polydnavirus, or symbiont, and can be detected by parasitoid females, a secondary clutch might even be expected to be larger in size than a primary clutch. For example, an increase in clutch size was observed for the gregarious ectoparasitoid, *Hyssops pallidus* (Askew) ovipositing on hosts previously parasitized by the solitary endoparasitoid *Ascogaster quadridentata* Wesmael (Zaviezo and Mills, 2001). However, once host resources begin to be extracted by larvae from a primary clutch, host quality would decline, and consequently, secondary clutches that result from larvicide would be expected to be smaller than the preceding primary clutches. The consequences of parasitism on the metabolic processes and nutritional quality of parasitized hosts remain poorly understood, particularly in the context of parasitoids that exhibit infanticide. Nonetheless, we anticipate that protein and lipid concentrations in the hemolymph of hosts with a primary clutch should be as high as for non-

parasitized hosts, provided that the parasitoid eggs have not yet hatched. This study aims to contribute to our knowledge of parasitoid behavioral ecology and clutch-size decisions in the context of infanticide and host quality.

1.3 MATERIALS AND METHODS

Host and parasitoid colonies and experimental conditions

Host and parasitoid colonies and all experimental observations were made at 25-27°C and a 16:8 h L:D photoperiod in a quarantine facility at the University of California, Berkeley. A laboratory colony of the light brown apple moth, *E. postvittana,* was established from individuals collected in 2007 from an invasive population in Santa Cruz, California. Prior to experimentation, we reared the host larvae on an artificial bean-based diet developed by Cunningham (2007). Upon pupation, *E. postvittana* pupae were transferred to ventilated 956 ml transparent polypropylene deli containers (Fabri-Kal, Kalamazoo MI) and provided with 10% honey-water with 0.1% sorbic acid via a 4 cm cotton wick in a 22 ml translucent polystyrene cup (SOLO, Highland Park, IL). Following oviposition, freshly laid egg masses were sterilized following Singh and Moore (1985) in a 5% formaldehyde solution for 20 min, soaked in water for 20 min, and air dried before being transferred to 95 ml translucent polystyrene cups (SOLO) filled one third with artificial diet.

A laboratory colony of *G. jacintae* was established from individuals collected in 2008 from the Yarra Valley, Australia where it is an indigenous parasitoid of *E. postvittana*. It was maintained on *E. postvittana* larvae fed on leaves of plantain (*Plantago lanceolata* L.). Mated *G. jacintae* females were kept individually in ventilated 500 ml transparent polypropylene deli containers (Pactiv, Lake Forest, Illinois) with streaks of a 50:50 honey-water solution applied to the sides twice each week. Five 4^{th}-5^{th} instar host larvae were provided weekly on excised plantain leaves with their stems inserted into 7 ml glass vials (Fisher Scientific, Pittsburg, PA) of water plugged with cotton wool. Individual parasitized larvae were transferred to 7 ml glass vials plugged with cotton wool to await adult parasitoid emergence. For experiments, naïve, mated *G. jacintae* females were kept individually in 35 ml glass vials (Fisher Scientific, Pittsburg, PA) and provided with streaks of honey-water solution until they were 2-3 days old.

Influence of host size on parasitoid handling time, clutch size, and brood performance

As in other clutch-size studies (Godfray, 1994), to determine the influence of host size on the handling time, clutch size and fitness consequences of *G. jacintae*, we selected host larvae with as wide a range of fresh weights from among the instars that are susceptible to parasitism to avoid a consistent linkage between host size and age. We measured their fresh weight on a Sartorius GD503 microbalance (± 0.1 mg). Following Zaviezo and Mills (2001), host larvae were exposed to *G. jacintae* females individually in arenas consisting of two 50 ml glass vials (2.6 x 9.5 cm) connected by a copper tube (1.5 mm diameter and 40 mm in length) that was inserted through their plastic caps. Both host and parasitoid were placed into one glass vial and a honey-water solution was streaked onto the sides of both glass vials. The double glass vial arena reduced the likelihood of self-superparasitism by allowing a female parasitoid to move to the second empty glass vial once it had completed handling the host larva in the first vial. We

prepared digital images of each experimental female to estimate hind tibia length (mm) using ImageJ®, as a measure of parent female size (Godfray, 1994).

For each experimental female parasitoid we made observations every 5 min for a 3 h period to record time to host encounter, duration of attack, oviposition time, guarding time, total host handling time, and duration of host paralysis (all in min). We analyzed handling times for females that successfully oviposited eggs on a host. We defined time to host encounter as the time it took a female to find and initiate attack of a host larva. The duration of attack began with host encounter and lasted until oviposition, and included the time taken to temporarily paralyze a host and to host feed before oviposition. Oviposition time included the time it took a female parasitoid to mount a paralyzed host larva, lay a full clutch of eggs and dismount the host. Guarding time began once a female dismounted a host larva on which she had oviposited and ended when she moved through the copper tubing into the second glass vial. As our observations only lasted 3 h the guarding time continued beyond the observation period in some cases and so may represent an underestimate of the true guarding time. Total host handling time began at host encounter and was completed when a female exited the first glass vial through the copper tube and represents the sum of the duration of attack, oviposition time, and guarding time. The duration of host paralysis began with probing of a host larva by a female parasitoid to initiate paralysis, and ended at the first sign of host larval movement.

Parasitoid oviposition success was measured as a binary outcome, based on whether a female that encountered a host larva also oviposited within the 3 h observation period. All parasitized host larvae derived from the handling time observations were subsequently kept in 50 ml glass vials with fresh excised plantain leaves until the brood had completed their development and adult offspring had emerged. As measures of parasitoid performance we noted the clutch size (number of eggs laid by the parent female), development time from egg to adult emergence (in days), brood survivorship (from egg to adult emergence), and offspring sex ratio (percent female). A total of 93 parasitoid females were observed to encounter a host larva within the 3 h observation period, but levels of replication declined for subsequent behavioral events and measures of parasitoid performance and are included with the results below.

Occurrence and consequences of infanticide

To examine infanticide in *G. jacintae*, individual naïve secondary females were exposed to host larvae representing different infanticide treatments in 50 ml glass vials with an excised plantain leaf and streaks of honey-water solution. To generate the infanticide treatments, we exposed 4th and 5th instar host larvae individually to a first female parasitoid in glass vials with streaks of honey-water solution and checked every 24 h for the presence of a clutch of eggs. After oviposition was observed these first females were removed from the vials. Parasitoid eggs hatched on day 3 under the experimental conditions used, and so parasitized host larvae were maintained on excised plantain leaves for either 2 days for the ovicide treatments or 4-5 days for the larvicide treatment. For the ovicide treatments, parasitized host larvae supporting natural primary clutch sizes of either 1-2, 3-4 or 5-7 eggs were used. In contrast, only one clutch size (1-2 larvae) was used for the larvicide treatment, and generated by removing any extra parasitoid larvae from natural primary clutches to limit the variance in host feeding. In addition, non-parasitized host larvae of the same age without a primary clutch were used as controls. For all

treatments the host larvae were then exposed to a second female parasitoid for 24 h to monitor the occurrence and consequences of infanticide.

We verified the occurrence of infanticide and the laying of a secondary clutch of eggs by using the visual remains of removed larvae and eggs and digital images of the host larvae before and after exposure to the second female parasitoid. The consequence of infanticide on the clutch size and performance of secondary broods was monitored as in the previous experiment, with average hind tibia length of female offspring from 19 broods as an additional performance measurement. The level of replication for each of the infanticide treatments was as follows: 1-2 eggs (n = 20), 3-4 eggs (n = 27), 5-7 eggs (n = 17), 1-2 larvae (n = 16), control (n = 35). As in the previous experiment levels of replication declined for successive measures of brood performance and are presented in the results below.

Influence of host size and parasitism on host macronutrients

To examine the influence of host size and parasitism on the nutritional quality of *E. postvittana* larvae, protein and lipid concentrations of the hemolymph were analyzed using 5th instar larvae. To determine the effect of host size alone on protein and lipid concentrations in *E. postvittana* larvae we selected a range of sizes (based on fresh weight) of non-parasitized larvae, and used 38 and 11 individuals for the protein and lipid analyses respectively. As the duration of the 5th larval instar is 8-9 days, we determined macronutrient concentration 4 days post-molt to ensure that the molting process would not confound the relationship between host size and macronutrient concentration.

To examine the effect of parasitism by *G. jacintae* over time on protein and lipid concentrations in 5th instar host larvae, we used newly molted larvae for both non-parasitized (control) and parasitized treatments. Host larvae were placed individually in 50 ml glass vials with a fresh excised plantain leaf and a mated, naïve *G. jacintae* female and some streaks of honey-water solution were added to the parasitized treatment only. After 24 h the parasitoid females were removed and both parasitized and non-parasitized host larvae were maintained on excised plantain leaves in the glass vials. We monitored larval fresh weight and changes in protein and lipid concentrations in the hemolymph of host larvae at 1, 4 and 7 days post treatment. These time intervals were selected because parasitoid egg hatch occurred on day 3, parasitoid larvae pupated within 9-10 days, and non-parasitized 5th instar host larvae pupated within 8-9 days under the experimental conditions used. A total of 125 larvae (91 non-parasitized and 34 parasitized) were used for this experiment and 40, 53 and 32 were analyzed on days 1, 4, and 7 post treatment. As not all larvae were used for analysis of both macronutrients, specific levels of replication for each are included in the results.

For both the host size and parasitism experiments host larvae were first weighed using a Sartorius GD503 microbalance, and then surface sterilized by wiping with a 90% ethanol-soaked tissue prior to severing a proleg with a needle to collect hemolymph. We collected 2 µl of hemolymph with a microcapillary tube and placed it into 20 µl of ice-cold anticoagulant buffer (98 mM NaOH; 0.19 M NaCl; 1.7 mM EDTA; 41 mM citric acid, pH 4.5) (Strand et al., 1997). We centrifuged each sample (1000g for 2 min) to remove unwanted tissues and stored the supernatant at -20°C for further analysis (Salvador and Cônsoli, 2008). We modified the

macronutrient analysis protocols of Salvador and Cônsoli (2008) to accommodate preparation of samples in 96 flat-well polystyrene microplates (BD FALCON™) and estimation of colorimetric absorbance in a microplate reader (BioRad iMark™). We determined total protein concentration in the hemolymph samples using the Coomassie Plus reagent (Coomassie Plus Protein, Pierce Biotechnology, Inc., Rockford, IL) and bovine serum albumin (Sigma-Aldrich A3803) as a standard, and measured absorbance at 595 nm. We determined the lipid concentration in the hemolymph using the vanillin reagent and vegetable oil as a standard. Hemolymph supernatant and standards were first transferred to glass vials and evaporated at 100 °C along with 200 µl chloroform: methanol (1:1). Subsequently we added 100 µl sulfuric acid and incubated the samples at 100 °C for 10 min. Absorbance was read at 590 nm both before and 5 min after adding 100 µl vanillin reagent and lipid concentration was determined from the difference in the two absorbance readings. We used duplicates for all samples and standards in the microplates to control for any pipetting error.

Statistical analysis

All statistical analyses were carried out using R (R Development Core Team, version 3.0.2, 2013) and were primarily based on generalized linear models (GLMs). To analyze the influence of host size on parasitoid handling time, clutch size, and brood performance, host larval fresh weight and female parasitoid size (hind tibia length) were included as fixed factors in GLM models for each measurement variable. For the consequences of infanticide experiment, primary and secondary clutch sizes from the same host larvae were first compared using a paired Wilcoxon signed-rank test. The various measures of brood performance of secondary clutches were then analyzed using GLMs with infanticide treatment, host larval fresh weight, and parent parasitoid female size as fixed factors. For the experiment on macronutrient concentrations in host larvae, we first analyzed protein and lipid concentrations in non-parasitized host larvae using general linear models (LMs) with larval fresh weight as the only fixed factor. Subsequently, ANCOVA was used to examine differences in the responses of macronutrient concentrations to treatment (control versus parasitized larvae) and to time (days post treatment) and host larval fresh weight ($\log_e$ transformed) as continuous explanatory variables.

GLM error distributions were selected to best represent the measurement variables analyzed (gaussian for continuous variables, Poisson for counts, binomial for proportions), quasi distributions were used when overdispersion occurred, and standard link functions were used in each case. Full models that included all explanatory variables and two-way interactions were used initially, and stepwise model simplification was performed manually using likelihood-ratio tests (χ^2) in the absence of overdispersion and *F* tests to incorporate an empirical scale parameter in the presence of overdispersion (Crawley, 2013). We ensured that error distributions and homogeneity were appropriate by inspecting residual, standardized deviance, and normal quantile plots (Crawley, 2013). As two-way interactions did not make a significant contribution to any of the GLMs, only the individual fixed factors are considered in the results, whereas interactions are presented in addition to the fixed factors for ANCOVA analyses of the effects of treatment, time post treatment and host larval fresh weight on host macronutrients.

1.4 RESULTS

Influence of host size on parasitoid handling time, clutch size, and brood performance

Mean time spent by *G. jacintae* on each of the successive host larval handling events, and the effects of host larval fresh weight and female parasitoid size on these events are presented in Table 1. Oviposition and guarding times contributed the most to the total handling time, and guarding times increased significantly with host larval fresh weight. Only 15% of the individuals that were monitored for total handling time took longer to exit the glass vial than the 3 h observation period. There was no effect of host larval fresh weight or parasitoid size on the duration of these events with the exception of oviposition time which increased with host larval fresh weight and the duration of host paralysis which decreased with parasitoid size (Table 1).

Oviposition success (65%) did not vary significantly with host larval fresh weight or parasitoid size (GLM binomial, $n = 93$, $\chi^2_1 = 0.26$, $P = 0.61$; $\chi^2_1 = 0.22$, $P = 0.65$ respectively). Clutch size was not affected by parasitoid size, but did increase significantly with host larval fresh weight (Fig. 1a, Table 2). For subsequent brood performance mean survivorship was 68% (± 31% SD), mean sex ratio was 79% female (± 21% SD), and neither were significantly influenced by host larval fresh weight or by parasitoid size (Table 2). Mean development time (oviposition to adult emergence) was 18.3 days (± 1.2 SD) and decreased significantly with an increase in both host larval fresh weight and parasitoid size (Table 2).

Occurrence and consequences of infanticide

Of the 80 *G. jacintae* presented with hosts previously parasitized with a primary clutch, 81% successfully removed all eggs or larvae from the first parasitoid and oviposited their own secondary clutch of eggs. Removal of eggs from their own secondary clutch was very rare for parasitoids committing infanticide, and only occurred when left with a host for more than 24 h. Of the 15 parasitoids that did not oviposit on a previously parasitized host, five partially removed the eggs or larvae from the primary brood. These parasitoids were not used for further analysis, but it was noted that the remaining eggs and larvae from the primary broods survived to adult emergence.

In comparing primary and secondary clutch sizes from the same individual host larvae (excluding the control treatment), second females oviposited a significantly larger clutch of eggs than first parasitoids for infanticide treatments with 1-2 eggs or 3-4 eggs (Paired Wilcoxon signed-rank test, $V = 88.5$, $n = 15$, $P = 0.02$ and $V = 94.5$, $n = 22$, $P = 0.05$ respectively). However, there was no significant difference between the clutch sizes of first and second parasitoids for infanticide treatments with 5-7 eggs or 1-2 larvae ($V = 46.5$, $n = 15$, $P = 0.46$ and $V = 22.0$, $n = 12$, $P = 1.0$ respectively).

When analyzed alone, the secondary clutch size dataset showed a significant increase in clutch size with host larval fresh weight (Fig. 1b) that was not affected by infanticide treatment (including the control treatment) or the size of the second parasitoid (Table 2). Thus the clutch size response of *G. jacintae* to host size was the same for both primary (Fig. 1a) and secondary (Fig. 1b) clutches. Brood survivorship from secondary clutches was not significantly influenced

by host larval fresh weight or by parasitoid size, but varied significantly with infanticide treatment (Table 2). Brood survivorship was greater for secondary clutches in the 1-2 eggs and 3-4 eggs treatments compared to the controls (a primary clutch on host larvae of the same age), with the 5-7 eggs and 1-2 larvae treatments being intermediate in brood survivorship (Fig. 2). As found from the initial experiment on primary clutches, development time of secondary clutches decreased with increasing parasitoid size and host larval fresh weight (marginal effect), but was also influenced by infanticide treatment (Table 2). Development time was shorter for the 5-7 eggs treatment (16.00 ± 2.20 SD) compared to all other treatments (18.27 ± 1.47 SD) with the exception of the 1-2 larvae treatment where it was also slightly shorter (17.00 ± 1.22 SD) (stepwise deletion, GLM, gaussian (log), $F_{3,60} = 29.89$, $P = 0.004$; $F_{1,60} = 2.43$, $P = 0.28$ respectively). The sex ratio (77% ± 22% SD) and size of female offspring (0.42 ± 0.07 SD) from secondary clutches were not significantly influenced by parasitoid size, host larval fresh weight, or infanticide treatment (Table 2).

Influence of host size and parasitism on host macronutrients

To determine whether host size alone can influence the nutritional quality of *E. postvittana* larvae for *G. jacintae* we first analyzed non-parasitized 5th instar larvae. Both protein (LM, $F_{1,37} = 21.27$, $P < 0.001$) and lipid (LM, $F_{1,10} = 9.27$, $P = 0.01$) concentration increased significantly with host larval fresh weight for non-parasitized larvae four days post molt (Fig. 3). We then compared the nutritional quality of similar sized control and parasitized 5th instar host larvae over a period of 7 days. A significant interaction between treatment and time for host larval fresh weight (LM, $F_{1,123} = 54.8$, $P < 0.001$) indicated that the two treatments differed in slope; control larvae continued to grow and gain weight, whereas following parasitism, parasitized larvae declined in fresh weight (Fig. 4a). For the analysis of protein concentration in larval hemolymph, there were significant interactions between host larval fresh weight and both time ($F_{1,117} = 15.82$, $P < 0.001$) and treatment ($F_{1,117} = 6.81$, $P = 0.01$). These interactions resulted from a strong increase in protein concentration over time as control larvae grew in fresh weight (Fig, 4a,b and Fig 3a) together with a weak increase in protein concentration over time despite a decline in the fresh weight of parasitized larvae (Fig. 4a,b). Consequently, while protein concentration was initially higher in parasitized larvae it dropped below that of control larvae soon after the timing of parasitoid egg hatch (day 3). For the analysis of lipid concentration in larval hemolymph, there were no significant interactions among host larval fresh weight, time, and treatment ($F_{3,47} = 0.65$, $P = 0.59$), no effect of host larval fresh weight ($F_{1,51} = 0.20$, $P = 0.66$), but significant effects of both treatment ($F_{1,52} = 18.81$, $P < 0.001$) and time ($F_{1,52} = 11.96$, $P = 0.001$). The absence of an interaction between treatment and time indicated that lipids increased at the same rate (no difference in slope) for both control and parasitized larvae and the significant effect of treatment indicated that lipid concentration in parasitized larvae was higher over the entire post treatment period than for control larvae (Fig. 4c).

1.5 DISCUSSION

Clutch size, and consequently oviposition time, of the gregarious ectoparasitoid *G. jacintae* was found to increase with larval fresh weight of its host *E. postvittana* in a similar way to that described for the related species *G. nephantidis* (Muesebeck) (Hardy et al., 1992). The allocation of more eggs to larger hosts is a common pattern that has been observed repeatedly for

gregarious ectoparasitoids (Godfray, 1994; Zaviezo and Mills, 2000; Wang et al., 2008; Koppik et al., 2014). In addition, our observations indicated that the host handling time for *G. jacintae* was short and that parent females readily committed infanticide of conspecific broods, laying larger secondary clutches in two out of three ovicide treatments, which subsequently experienced greater survivorship and in some cases a shorter development time than primary clutches. From our analysis of macronutrient concentrations in parasitized and non-parasitized host larvae it appears that the difference in performance of primary and secondary clutches may be related to an increase in the protein and lipid concentration of the host larval hemolymph following parasitism. In addition, some anomalous effects of parasitoid female size on the duration of host larval paralysis and the development time of both primary and secondary parasitoid broods were observed during the study, but we are currently unable to explain the significance of these observations.

From our direct observations of host handling by *G. jacintae*, adult females guarded their brood for an average of only 66 min post oviposition, a brief period that seems unlikely to provide much protection for the developing brood from either intraspecific or interspecific competitors. While this represents a slight underestimate of the true guarding time, due to the 3 h limit for our experimental observations, the majority of female parasitoids left their hosts before the end of the observation period and so the level of underestimation is likely small. Variation in the duration of brood guarding and propensity for infanticide among bethylid parasitoids may be influenced by life history strategies linked to the rarity of their hosts (Godfray, 1994), or by the likelihood of interspecific competition (Hardy and Blackburn, 1991). The fitness costs of brood guarding may be related to the rarity of hosts, such that species with a greater lifetime fecundity use hosts that are typically more common and spend less time guarding individual broods (Mayhew, 1997), but readily commit infanticide under conditions when hosts are less abundant. However, *G. legneri* Gordh has a high fecundity of 156 eggs (Laumann et al., 2000), but guards its larval brood for 5-6 days, and commits both ovicide and larvicide (Bentley et al., 2009). Similarly, *G. nephantidis* has a fecundity of 117 eggs (Sreekanth and Muralimohan, 2013), guards its brood until pupation (Hardy and Blackburn, 1991; Goubault et al., 2007), and yet commits ovicide only (Hardy & Blackburn, 1991). In contrast, *G. indicus* Ashmead has a low fecundity of 30 eggs, but guards its brood only until egg hatch, and commits ovicide only (Takasu & Overholt, 1998). In this context, *G. jacintae* has an intermediate fecundity of 64 eggs (unpublished data), a minimal level of brood guarding, and readily commits both ovicide and larvicide. Thus brood guarding and infanticide vary widely among bethylid species and appear not to be influenced by rarity of hosts (fecundity) or intraspecific competition (vulnerability to infanticide). In contrast, brood guarding may be more important for protection against interspecific rather than intraspecific competitors, as appears to be the case for *G. nephantidis* (Hardy and Blackburn, 1991; Venkatesan et al., 2009).

Infanticide may also be adaptive for *G. jacintae* for reasons other than competition and the potential rarity of non-parasitized hosts. For example, encapsulation rates for secondary clutches were found to be lower than for primary clutches of *Metaphycus flavus* (Howard) in soft scale hosts (Tena et al., 2008). In addition, Takasu and Hirose (1991) proposed that parasitized hosts were preferred over non-parasitized hosts by *Ooencyrtus nezarae* Ishii due to the presence of pre-drilled oviposition holes in host eggs that reduced the handling time for a second female. While a reduction in encapsulation or host handling time would not be applicable to *G. jacintae*,

other potential advantages of infanticide include nutrients gained from consumption of eggs or larvae from a primary clutch (Goubault et al., 2007), and improved nutritional quality from previous parasitism.

One of the important findings from this study was that for the same host individuals, secondary clutches of *G. jacintae* were significantly larger than primary clutches for the 1-2 eggs and 3-4 eggs ovicide treatments. In addition, these secondary broods experienced greater survivorship and in some cases a shorter development time, although other aspects of performance such as sex ratio and offspring size did not differ from that of primary broods. This contrasts with a study of the bethylid *L. pedatus*, which showed that there was no difference between primary and secondary clutch sizes and that offspring size was smaller from secondary than from primary broods (Mayhew, 1997). However, a study by Zaviezo and Mills (2001) demonstrated that clutch size of the eulophid ectoparasitoid *H. pallidus* was twice as large, with twice as many females of typical size, on hosts previously parasitized by the braconid endoparasitoid *A. quadidentata*. This latter study suggested that previous parasitism might have improved the nutritional status of host larvae for *H. pallidus*. Our analysis of protein and lipid concentrations in *E. postvittana* larvae parasitized by *G. jacintae* also suggests that the mechanisms permitting greater clutch size and brood survivorship for secondary clutches could be nutritional.

Protein concentrations in the hemolymph of parasitized *E. postvittana* larvae continued to increase at a slow rate over time despite a reduction in host larval fresh weight. However, the rate of increase was less than observed for non-parasitized host larvae that grew substantially in fresh weight such that protein concentrations in parasitized larvae fell below that of non-parasitized larvae after day 3. A similar increase in protein concentration of the hemolymph over time has been observed for parasitized aphids (Pennacchio et al., 1995). In addition, lipid concentrations in the hemolymph of parasitized larvae were both consistently higher than in non-parasitized larvae and increased over time at the same rate as in non-parasitized larvae. The elevated lipid concentrations are consistent with similar findings by Nakamatsu and Tanaka (2004) for the gregarious ectoparasitoid, *Euplectrus separatae* Kamijo. In this case, both natural and artificial injection of parasitoid venom increased lipid concentrations in the larval hemolymph of *Pseudaletia separata* Walker. As the majority of adult parasitoids are incapable of synthesizing lipids (Visser et al., 2010), their acquisition from hosts during larval development is extremely important for the realized fecundity of adult females. Thus the greater protein and lipid concentrations in the hemolymph of previously parasitized *E. postvittana* larvae may provide a valuable advantage to *G. jacintae* in enhancing the life history performance of secondary clutches derived from ovicide events. Since primary clutches were removed by second females before any of the eggs had hatched, first females appear to have primed the host nutritionally during host handling, perhaps through the use of venom for temporary host paralysis. If detectable by an infanticidal female, the improved nutritional status of previously parasitized hosts could provide the cue for laying a larger secondary clutch on the same host individual than the first female. Whether a second parasitism event via infanticide leads to a further additive effect on lipid and protein concentrations in host hemolymph, beyond that observed in response to a first parasitism event, was not tested in the current study. Thus it remains unknown whether the increased survivorship of secondary broods was due to nutritional priming of the host by the first female or to additional priming during the second parasitism event.

In conclusion, as a gregarious ectoparasitoid, *G. jacintae* appears to base its clutch size decisions on both the nutritional quality and size of its host. This study is the first to provide evidence that improved nutritional quality could be an important benefit of infanticide for a gregarious ectoparasitoid, and one that may be responsible for the larger clutch size and enhanced performance observed among secondary ovicidal clutches of *G. jacintae*.

1.6 TABLES

Table 1. Mean time (min) spent by *Goniozus jacintae* in encountering and handling larvae of *Epiphyas postvittana*, duration of host paralysis, and results of generalized linear models (gaussian errors) to test for effects of host larval fresh weight and parasitoid size. Significant effects are shown in bold.

Model Factor	**Mean ± SD (min)**	***F***	**d.f.**	***P***
Time to host encounter	28.85 ± 39.39			
Host weight		0.51	1, 58	0.48
Parasitoid size		0.48	1, 57	0.49
Duration of attack	28.80 ± 19.99			
Host weight		1.11	1, 49	0.30
Parasitoid size		0.01	1, 48	0.93
Oviposition time	47.20 ± 38.05			
Host weight		5.33	1, 38	**0.03**
Parasitoid size		1.99	1, 37	0.17
Guarding time	66.05 ± 49.17			
Host weight		0.00	1, 18	0.95
Parasitoid size		0.00	1, 17	0.97
Total handling time	118.60 ± 62.39			
Host weight		0.05	1, 17	0.82
Parasitoid size		0.49	1, 18	0.49
Duration of paralysis	65.72 ± 27.15			
Host weight		0.27	1, 8	0.62
Parasitoid size		5.08	1, 9	**0.05**

Table 2. Statistical analysis of the brood performance characteristics of primary clutches of *Goniozus jacintae* from the host size experiment, and of secondary clutches from the infanticide experiment. Significant effects are shown in bold.

Model		**Primary clutch**				**Secondary clutch**		
Factor	**n**	**GLM family**	**d.f.**	***P***	**n**	**GLM family**	**d.f**	***P***
Clutch size	60	Poisson, $\chi 2$			87	Poisson, $\chi 2$		
Host weight		**12.94**	**1**	**< 0.001**		**19.29**	**1**	**< 0.001**
Parasitoid size		0.61	1	0.44		0.58	1	0.45
Infanticide treatment						6.62	4	0.16
Brood survivorship	38	quasi-binomial, *F*			87	quasi-binomial, *F*		
Host weight		0.68	1, 34	0.53		2.73	1, 81	0.1
Parasitoid size		0.90	1, 35	0.35		2.21	1, 80	0.31
Infanticide treatment						**23.84**	**4, 82**	**0.03**
Development time	37	gaussian (log), *F*			67	gaussian (log), *F*		
Host weight		**7.18**	**1, 34**	**0.01**		3.82	1, 60	0.06
Parasitoid size		**6.65**	**1, 34**	**0.01**		**7.66**	**1, 60**	**0.01**
Infanticide treatment						**3.84**	**4, 60**	**0.01**
Offspring sex ratio	26	binomial, $\chi 2$			47	binomial, $\chi 2$		
Host weight		0.12	1	0.73		0.01	1	0.93
Parasitoid size		0.00	1	0.98		0.39	1	0.53
Infanticide treatment						0.69	4	0.95
Offspring size					19	gaussian (log), *F*		
Host weight						1.11	1, 18	0.31
Parasitoid size						0.06	1, 14	0.82
Infanticide treatment						0.42	1, 15	0.74

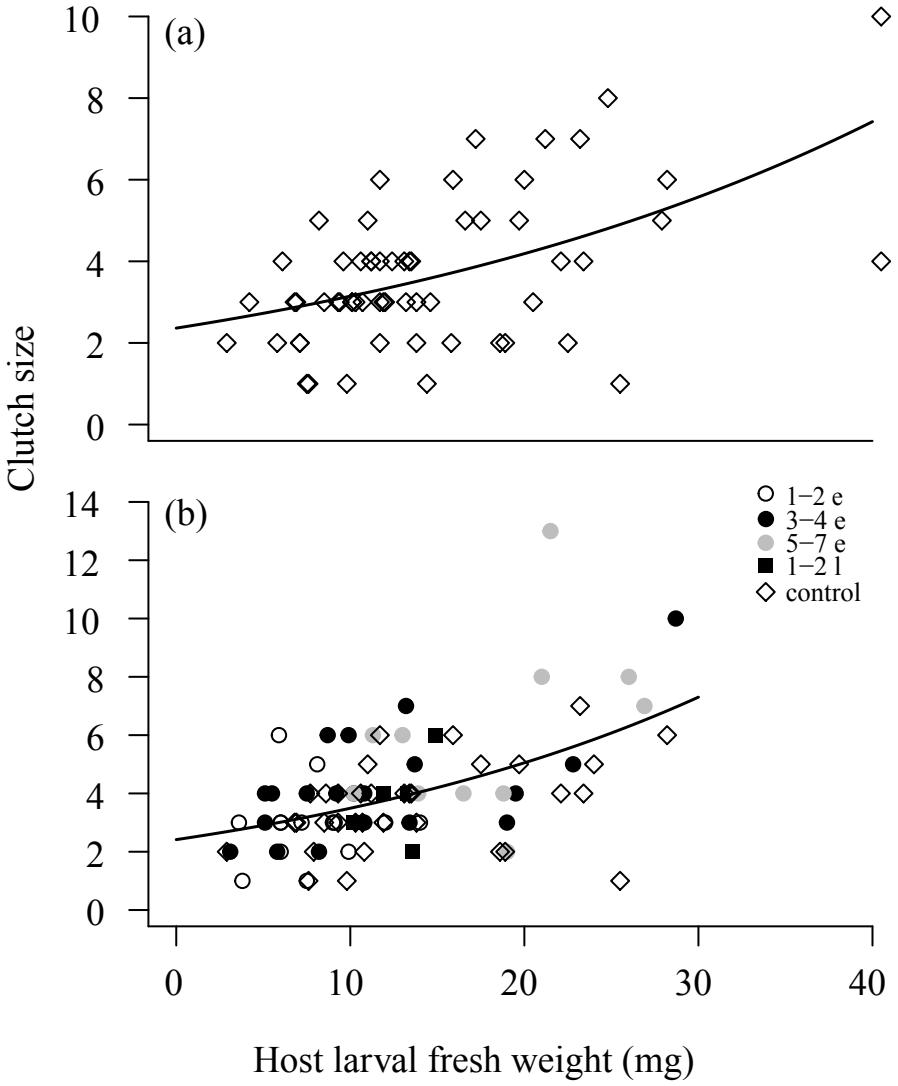

Figure 1. Clutch size response of *Goniozus jacintae* in relation to larval fresh weight of the host, *Epiphyas postvittana* for (a) primary clutches of first females from the host size experiment [y = exp(0.03*x + 0.86)], and (b) for secondary clutches of second females from the infanticide experiment [y = exp(0.04*x + 0.88)]. All successful ovipositions by the second female took place after complete removal of eggs (ovicide) or larva (larvicide) from the first female.

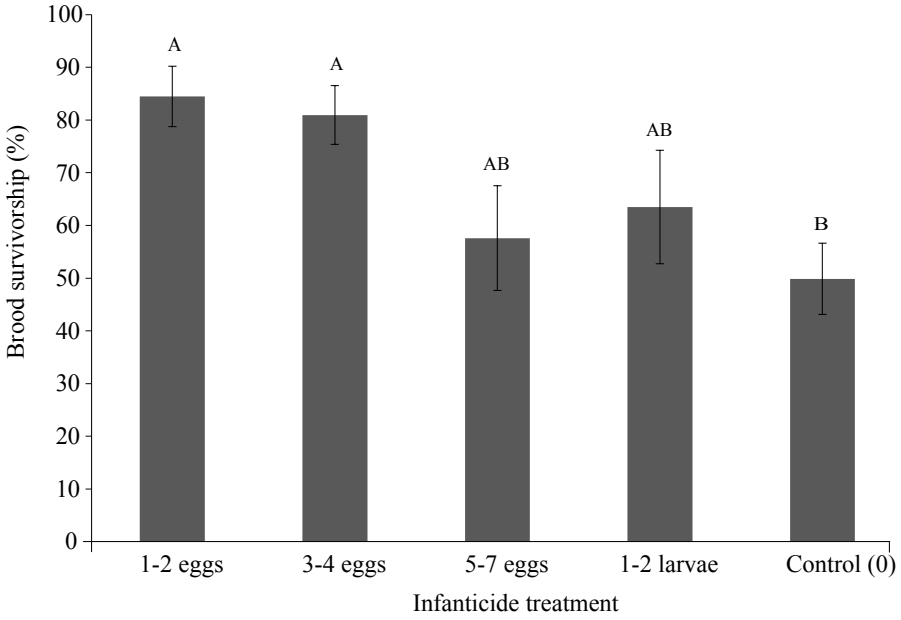

Figure 2. Mean (± SE) brood survivorship of secondary clutches laid by *Goniozus jacintae* in relation to infanticide treatment; treatments with different letters are significantly different using a GLM (quasi-binomial) with stepwise deletion.

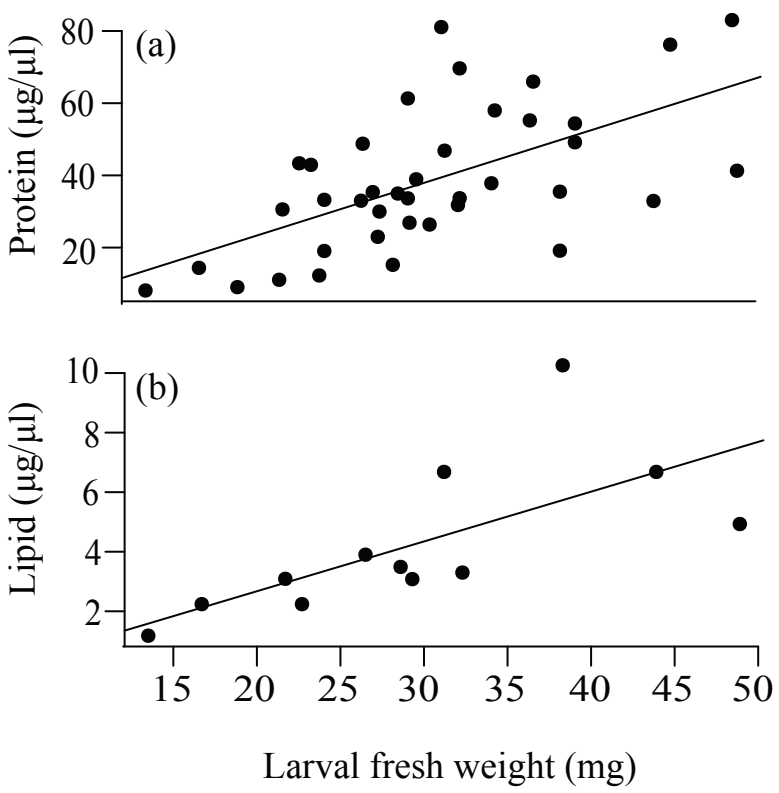

Figure 3. Protein (a), and lipid (b) concentrations (µg/µl) in the hemolymph of non-parasitized 5th instar larvae of *Epiphyas postvittana* (four days post-molt) in relation to fresh weight (protein y =1.46x – 6.08, lipid y = 0.17x – 0.67).

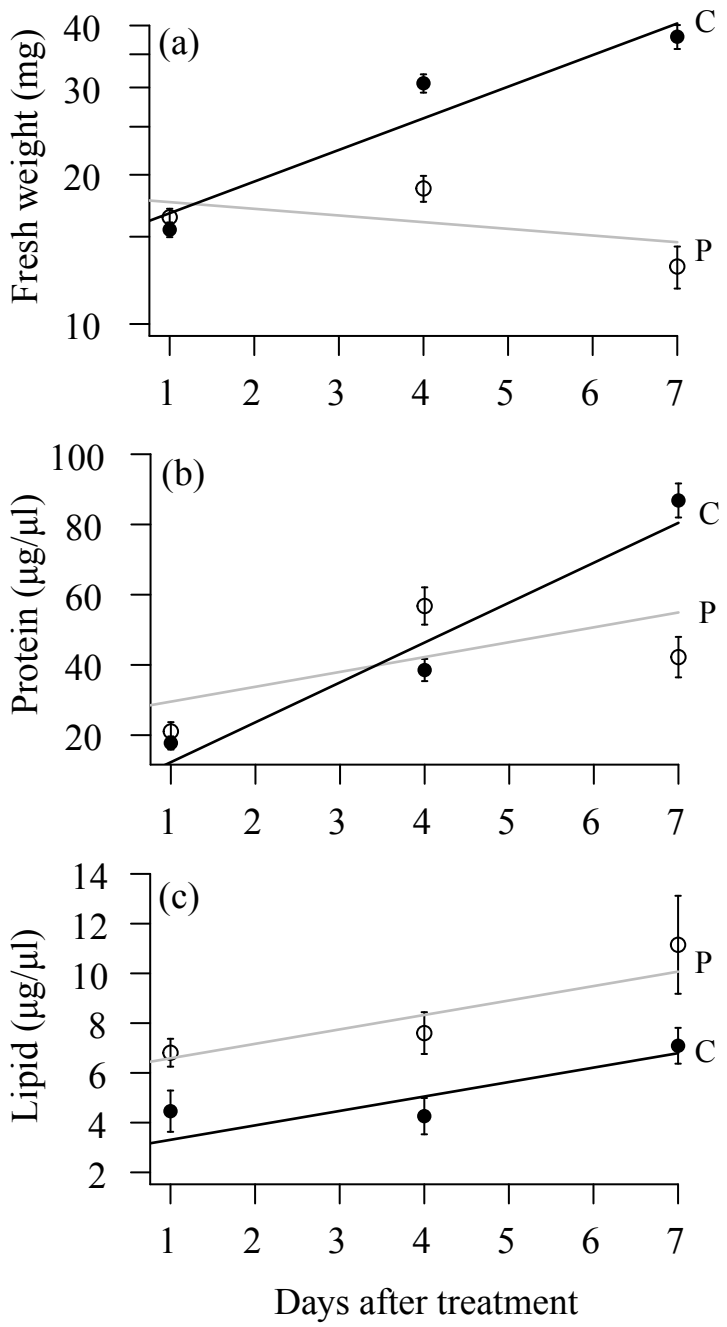

Figure 4. Mean (± SE) larval fresh weight (a), and protein (b) and lipid (c) concentrations in the hemolymph of 5th instar *Epiphyas postvittana* as non-parasitized controls (C) and as hosts parasitized by *Goniozus jacintae* (P) in relation to days after treatment. Fitted lines are (a) control y = exp (0.147x + 2.67), parasitized y = exp (-0.031x + 2.89); (b) control y = 11.36x + 0.94, parasitized y = 4.23x + 25.31; and (c) control y = 0.58x + 2.73, parasitized y = 0.58x + 6.01.

Figure S1.1. Female *Goniozus jacintae* performing larvicide on offspring from a different female (on the larval host, *Epiphyas postvittana*).

CHAPTER 2

PATHOGENICITY, MORPHOLOGY, AND CHARACTERIZATION OF A *NOSEMA FUMIFERANAE* ISOLATE (MICROSPORIDIA: NOSEMATIDAE) FROM THE LIGHT BROWN APPLE MOTH, *EPIPHYAS POSTVITTANA* (LEPIDOPTERA: TORTRICIDAE) IN CALIFORNIA

2.1 ABSTRACT

We recently discovered infections by a microsporidium closely related to *Nosema fumiferanae* in field populations of the light brown apple moth, *Epiphyas postvittana* (Walker) (Lepidoptera: Tortricidae), in the San Francisco region of California. *E. postvittana* originates from Australia and was first detected in California in 2006; therefore, our aim was to identify and determine the origin of the *Nosema* isolate. We characterized the pathogenicity, transmission pathways, and ultrastructure of this new *Nosema* isolate. In addition, we sequenced fragments of commonly used genetic markers (ITS, SSU, and RPB1), and examined the phylogenetic relationships between the *Nosema* isolate and other microsporidian species commonly found in lepidopteran hosts. The pathogenicity of the *Nosema* isolate was investigated by infecting second instar larvae of *E. postvittana*. Larval and pupal survivorship were reduced by 7% and 13% respectively, and pupation occurred 1-2 d later in infected individuals than in healthy individuals. Emerging infected females died 5 d earlier than healthy females, and daily fecundity was 22% lower. Hatch rate also was 22% lower for eggs oviposited by infected females. Vertical transmission was confirmed; spores were present in 68% of egg masses and 100% of the surviving larvae from infected females. Ultrastructure images, together with sequences from selected genetic markers, confirmed the *Nosema* isolate to be a member of the *Nosema fumiferanae* species complex (*Nosema fumiferanae postvittana* subsp. n.). The association of this pathogen with *E. postvittana* contributes further to the biotic resistance that *E. postvittana* has experienced since its introduction to California.

2.2 INTRODUCTION

Microsporidia are a diverse group of intracellular pathogens suggested to belong to a newly erected superphylum, Opisthosporidia, a deep-branch clade of Holomycota related to the Fungi (Karpov et al., 2014; Keeling, 2014) and comprise over 185 described genera and over 1300 species that infect protists, invertebrates and vertebrates, including humans (Solter et al., 2012a; Vavra and Lukes, 2013). There are more than 150 described species in the genus *Nosema* (Microsporidia: Nosematidae) that are associated with 12 different orders of insects (Becnel and Andreadis, 2014). The type species of the genus, *Nosema bombycis* (Nägeli) was the first member of the Microsporidia to be described, and is the causal agent of pébrine disease in the silkworm, *Bombyx mori* L. (Vavra and Lukes, 2013; Becnel and Andreadis, 2014). In addition to the impact of *N. bombycis* in sericulture, several microsporidian species have expanded their geographic range either as accidental or deliberate introductions. *N. ceranae* Fries, suggested to originate from *Apis cerana* F. in Asia (Fries et al., 1996; Gomez-Moracho et al., 2015), has been found in Europe, North America, and South America where it infects *A. mellifera* L. and bumble bees (Graystock et al., 2013). *N. tyriae* Canning was accidentally introduced from Europe to North America along with the deliberate introduction of its host, the cinnabar moth, *Tyria jacobaeae* (L.) (Hawkes, 1973), and *N. lymantriae* Weiser and *Vairimorpha disparis* (Timofejeva) were deliberately introduced from Europe to North America in trial studies for biological control of the gypsy moth, *Lymantria dispar* (L.) (Hajek and Delalibera, 2010).

In 2013, we discovered an infection by a microsporidium resembling a *Nosema* species in a laboratory colony and in field populations of the light brown apple moth, *Epiphyas postvittana* (Walker) (Lepidoptera: Tortricidae), in the San Francisco region of California. *E. postvittana* is an exotic leaf-roller native to Australia, with a host plant range that includes over 500 plant species (Suckling and Brockerhoff, 2010), and was originally detected in California in 2006 (Brown, 2007). Since its introduction, *E. postvittana* has been effectively colonized by a range of resident insect parasitoids, some of which are contributing significant biotic resistance to the spread of this new invader (Wang et al., 2012; Bürgi and Mills, 2014; Bürgi et al., 2015). Thus, it was of interest to characterize this microsporidium and determine its pathology in *E. postvittana*. Initial sequence information indicated that this new isolate resembled *Nosema fumiferanae* (Thompson).

N. fumiferanae is a well-characterized microsporidium of the Eastern spruce budworm *Choristoneura fumiferana* (Clem.) (Lepidoptera: Tortricidae). *C. fumiferana* is one of the most destructive forest defoliators in North America with its common host plants including balsam fir, *Abies balsamea* (L.) and white spruce, *Picea glauca* (Moench) Voss (Zhao et al., 2014). During an outbreak, up to 80% of *C. fumiferana* can be infected with *N. fumiferanae* (Thomson, 1960b), but more generally it is a low virulence pathogen with a prevalence that varies between 10-50% (Eveleigh et al., 2012). Transmission of *N. fumiferanae* occurs horizontally through consumption of spores (Campbell et al., 2007) and vertically from the parent female to her offspring (Thomson, 1957; Bauer and Nordin, 1989; van Frankenhuyzen et al., 2007). The midgut of the host is the principal site of infection, and the spores eventually spread to the Malpighian tubules, fat body, silk glands, epidermis, gonads, hind gut and nerve tissue (Thomson, 1955). Infection can result in lethal or sublethal effects depending on spore load and age of the host, with younger larvae being more susceptible to lethal effects than older larvae (Thomson, 1955). Larvae of *C.*

fumiferana with sublethal *N. fumiferanae* infections have prolonged development to pupation, and reduced pupal weight, fecundity and adult longevity (Thomson, 1957). In the laboratory, spores of *N. fumiferanae* have also been found in the midgut lumen of two parasitoids of *C. fumiferana*: *Apanteles fumiferanae* Vier. and *Glypta fumiferanae* (Vier.) (Thomson, 1958a). In addition, laboratory tests have shown that *N. fumiferanae* can have pathogenic effects in other lepidopteran hosts, including the oblique-banded leafroller, *Choristoneura rosaceana* (Harris) (Cossentine and Gardiner, 1991) and the western tent caterpillar, *Malacosoma californicum pluviale* (Dyar) (Wilson, 1974). However, currently *N. fumiferanae* has only been confirmed from field populations of *C. fumiferana*.

Historically, identification of *N. fumiferanae* has been based on host association, pathological and morphological characteristics such as the primary site of infection, spore size, type of cell division, number of nuclei per cell, and ultrastructural details (Thomson, 1955; Percy, 1973). However, detailed morphological descriptions of microsporidia remain limited as they require ultrastructural images using transmission electron microscopy (TEM) (Solter et al., 2012b). As *Nosema* species often exhibit limited pathological and morphological differences, ribosomal DNA (rDNA) sequences recently have been used for more accurate identification (Solter et al., 2012b). Although a draft genome of *N. bombycis* and additional proteomic data are available for optimization of molecular markers (Pan et al., 2013), only rDNA loci have consistently been used for differentiating microsporidian species. These loci include the internal transcribed spacer (ITS) and the small subunit (SSU) rRNA gene, which can be used to distinguish the 'true' *Nosema* group from other microsporidia (Huang et al., 2004; Solter et al., 2012b). An additional potentially useful single copy nuclear marker is RNA polymerase II subunit (RPB1) (Cheney et al., 2001). This locus has proved useful in supporting higher level relationships, e.g. between microsporidia and fungi (Hirt et al., 1999), as well as for identification of several microsporidian genera and species (Cheney et al., 2001; Vavra et al., 2006a; Gisder and Genersch, 2013; Luo et al., 2014).

Here, we characterize the pathogenicity, transmission pathways, and morphology of a *Nosema* isolate from *E. postvittana, Nosema fumiferanae postivittana* subsp. n. (hereafter *N. fumiferanae postvittana*). We also sequenced three commonly used genetic markers (ITS, SSU, and RPB1) to identify the isolate and compare it to other *Nosema* species from lepidopteran hosts. We include further TEM imaging of *Nosema fumiferanae* from *C. fumiferana* (hereafter *N. fumiferanae*); previous ultrastructural images did not include the mature spore stage (Percy, 1973). We compare the ultrastructure of the two *N. fumiferanae* isolates, and relate the sequence and ultrastructure characteristics to other microsporidia in the *N. bombycis* group.

2.3 MATERIALS AND METHODS

Microsporidia isolates

Microsporidian spores were isolated in 2013 from a laboratory colony of *E. postvittana* that was initially established from larvae collected in Santa Cruz, California in 2007 and supplemented with adult females collected from Richmond and Berkeley, California in 2013. Spores of *N. fumiferanae* were originally collected from *C. fumiferana* in Sault Ste. Marie, Ontario, Canada in 2001 and stored in liquid nitrogen at -80°C prior to use.

Host colonies and spore production

Infected and healthy host colonies were maintained at 20°C, a 16:8 h L:D photoperiod, and a relative humidity above 60%, and all experiments were conducted under the same conditions. The healthy colony was located in an insectary and separate growth chambers were used for the infected colony and for all experiments. We used 10% bleach to sterilize all tools, containers and surfaces prior to use. An uninfected (healthy) laboratory colony of *E. postvittana* was established from egg masses donated by USDA-APHIS from a separate colony of the same original source population from Santa Cruz, California. Larvae were reared on an artificial bean-based diet developed by Cunningham (2007). Upon pupation, an equal number of pupae of each sex were transferred to 956 ml ventilated plastic oviposition cups. Prior to adult emergence, the cups were provided with 10% honey-water with 0.1% sorbic acid via a 4 cm cotton wick in a 22 ml plastic cup. Following oviposition, freshly laid egg masses were sterilized following Singh et al. (1985) in a 5% formaldehyde solution for 20 min, soaked in water for 20 min, and air dried in a sterile area before being transferred to 96-ml plastic cups containing approximately 30 ml artificial diet. We routinely evaluated larvae from the healthy colony to ensure that no microsporidian infections had occurred in the healthy insects.

An infected colony of *E. postvittana* was reared as above with the addition of 10^5 *N. fumiferanae postvittana* spores per ml mixed into the artificial diet. We utilized infected ultimate instar larvae to obtain pure spore suspensions of the isolate by homogenizing the larvae in DI water with a plastic pestle and filtering the homogenate through nylon mesh to remove host tissues and integument. We followed the 'triangulation method' of purification by Cole (1970) to further purify the homogenate. We used a hemocytometer and phase contrast microscopy (400x magnification) to count the number of spores per ml from individual larvae taken from the infected colony. We subsequently diluted the purified spore homogenate with DI water to obtain a concentration of 5 x 10^2 spores per µl.

Pathology and vertical transmission

To determine the effects of *N. fumiferanae postvittana* infection in *E. postvittana*, experimental second instar larvae in 22-ml plastic cups were starved for 24 h prior to providing a 2-mm cube of artificial diet with either 2 µl distilled water (healthy treatment) or 2 µl spore homogenate of *N. fumiferanae postvittana* (infected treatment). After 24 h when the 2-mm cube of diet had been completely consumed, we provided all larvae a fresh 1.5-cm cube of diet (no inoculum), and refreshed the diet cube every week. We recorded larval and pupal development time and survivorship, sex ratio (percent female) at pupation, pupal fresh weight, adult emergence, proportion of females that oviposited, per capita daily fecundity, and egg hatch. Pupae were weighed 24-48 h after pupation. Freshly eclosed females were placed with two healthy males in an oviposition cup and the date of female death was recorded. We collected all egg masses and stored them individually in sterile cups with moist cotton wicks to record both the number and the proportion of egg hatch. To confirm that healthy individuals remained uninfected, we monitored larval frass for the presence of spores from healthy and infected individuals at the time of pupation (or earlier if larval mortality occurred) by examining smears in 2 μl water on glass slides under phase contrast microscopy, 400x magnification.

We infected 57 second instar larvae with 10^3 *Nosema* spores as described above, and subsequently monitored their development until 22 surviving female moths emerged. These females were mated with healthy males and oviposition was monitored as described above. Following initial oviposition (day 1), each female was transferred to new oviposition cups on days 1, 5, and 9 to allow the collection of fresh uncontaminated egg masses on days 2, 6 and 10 of oviposition. The females were returned to their original oviposition cups on days when egg masses were not collected for evaluation. The number of eggs per egg mass was counted prior to microscopic examination to account for any correlation between egg mass size and spore presence and infection intensity. Half of the egg masses collected on days 2, 6, and 10 were examined immediately, and the other half were allowed to hatch. Five larvae from each egg mass were examined. Egg masses and larvae were smeared onto glass microscope slides in 2 μl of water. To determine whether spores were present, observations were made at 20 random fields of view at 400x magnification.

Tissues infected and morphology

To determine the progression of infection and occurrence of spores in different tissues of *E. postvittana*, we infected larvae just prior to molting to fifth instar with 10^3 spores of both microsporidian isolates and maintained them on artificial diet as described above. After molting to fifth instar, we dissected five larvae infected with the *N. fumiferanae postvittana* isolate every 24 h and rinsed freshly dissected tissues in 0.9% saline solution before smearing them on glass microscope slides under glass coverslips. We examined the tissues using 20 random fields of view under phase contrast microscopy at 400x magnification. We confirmed spore presence when necessary by staining the tissues with a modified Giemsa stain (Sigma-Aldrich) (Becnel, 2012) and examining the samples at 1000x magnification. We measured the length and breadth of 10 mature spores of both isolates with ImageJ® using an ocular scale at 1000x magnification. Measurements did not include the refractory spore border.

To compare the morphology of the two *Nosema* isolates with transmission electron microscopy (TEM), we adapted methods from Lange et al. (2009). We infected *E. postvittana* larvae and prepared tissues at different times to avoid any possible cross contamination. Although primary spores were observed in early stage infections, images for all stages were from late-stage infections and represented the second sporulation cycle (S2) (see Vavra et al., 2006b). For each isolate we dissected fifth instar larvae that had been infected in the second stadium and fixed infected mid-gut tissues for 1 h in 2% glutaraldehyde in 0.1M sodium cacodylate buffer, pH 7.2. The fixed tissues were rinsed in 0.1M sodium cacodylate buffer three times for 5 min and post-fixed in 1% aqueous osmium tetroxide and 1.6% potassium ferricyanide in 0.1M sodium cacodylate buffer for 30 min. Post-fixed tissues were rinsed three times with 0.1 M sodium cacodylate buffer (5 min rinses) followed by three 10-min rinses with DI water. The tissues were then en bloc-stained with 0.5% aqueous uranyl acetate for 30 min in the dark and rinsed a further three times in DI water (5 min rinses). After staining, the tissues were dehydrated through an ascending acetone series (7 min in each of 35%, 50%, 70%, 80%, 95%), followed by 100% acetone for three times (7 min each). The tissues were then infiltrated through three gradients (15 min in each of 2:1, 1:1, 1:2) of acetone: Epon resin (23.5 g Eponate 12, 12.5 g dodecenyl succinic anhydride, 14 g nadic methyl anhydride, and 0.75 ml dimethylbenzylamine as the

accelerator) followed by three times (15 min each) of pure resin. Tissues were agitated at all steps and embedded into molds with pure resin and accelerator at 60°C for 72 h. Thin 70 nm sections were cut on a Reichert-Jung Ultracut E ultramicrotome, stained with 2% aqueous uranyl acetate and lead citrate, and examined with a Tecnai 12 transmission electron microscope.

DNA extraction, sequencing and phylogenetic analysis

DNA was extracted from the *N. fumiferanae postvittana* isolates using the modified Chelex method (Walsh et al., 1991; Cordes et al., 2012). Purified spores (approximately 10^4-10^5) were added to 25 μl Chelex buffer composed of 10% Chelex 100 resin (BioRad), 5% tween20, and 1 μg/μl proteinase K, in a 200μl PCR tube. The Chelex/spore mixtures were vortexed for 15 s at maximum speed and transferred into a thermocycler (BioRad iCycler). The mixtures were treated at 56 °C for 120 min, 95 °C for 30 min, and 4 °C for 1 min. The tubes were then centrifuged at 13,000g for 10 min. Supernatants were stored at -20 °C until use.

We amplified fragments of three loci (ITS, SSU, and RPB1) that are commonly used for microsporidian species identification using standard PCR protocols. PCR reactions were conducted on a BioRad iCycler using the following conditions. After an initial denaturation step at 95 °C for 2 min, fragments were amplified using 40 cycles: 95 °C for 30 s, the fragment specific annealing temperature for 30 s (50 °C or 51 °C: Supplementary Table S2.1), and 72 °C for the fragment specific initial elongation time. A final elongation for 7 min at 72 °C was used. Primers and PCR conditions for amplification of each fragment are presented in Table S2.1. PCR reactions used 1unit (0.2 μl) Taq DNA polymerase with 5 μl standard buffer, 1 μl dNTP mix, 1μl DNA solution (supernatant from the Chelex method), and 1 μl of each 10 nM primer (100 nM in the case of the degenerate primers, Rpb1f1/Rpb1r1); in a 50μl reaction. PCR products were run on a 1.5% agarose gel electrophoresis with 1× TAE buffer and stained in ethidium bromide solution (1ppm). PCR products with a single band and meeting estimated amplicon sizes in the gel electrophoresis were cleaned using ExoSAP-IT, and sequencing was performed with an ABI 3730 DNA analyzer with BigDye using concentrations and protocols according to the manufacturer's instructions. Sequences were submitted to GenBank under accession numbers KT020736 and KT020735 for ITS-SSUrRNA and RBP1 respectively.

The ITS region of the reversed rRNA gene arrangement (LSU-ITS-SSU) found in the *N. bombycis* group tends to be highly polymorphic (Huang et al., 2004), particularly in the center of the ITS region (~200 bp) (Ironside, 2013). As the ITS region adjacent to LSU was 100% identical to all published *Nosema* species having the reversed rRNA arrangement we focused on the more variable region adjacent to SSU. In addition, because the primers for ITS and SSU amplified fragments that had overlapping sequences, these two regions were assembled into a single continuous sequence for the phylogenetic comparison (ITS-SSU).

The ITS-SSU and RPB1 fragments amplified from our *N. fumiferanae postvittana* isolate were compared to other available sequences from the genus *Nosema* and *Vairimorpha* that have been published in GenBank. Sequences were not consistently available for both markers and so the species representation differed for each marker (Table S2.3). An alignment for each locus was constructed using Clustal X version 2.0 (Larkin et al., 2007). The alignment was adjusted by eye and then truncated. Phylogenetic relationships between amplified and published sequences were

reconstructed based on maximum likelihood using MEGA version 6.0 (Tamura et al., 2013) for each alignment under the GTR model with estimated gamma distribution and proportion of invariable sites following the methods of James et al. (2006). Support for each node was estimated using 1,000 bootstrap replicates. To find a suitable outgroup for the ITS-SSU analysis, we noted that the non-coding region adjacent to the 5' end of *N. ceranae* SSU (Huang et al. (2008); GenBank accession # EF091879) shared 48-57% similarity with the non-polymorphic ITS region of *Nosema* species that have the reversed rRNA gene arrangement (Fig. S2.1). We hypothesized that the fragments are homologous and used *N. ceranae* as the outgroup species in the phylogenetic analysis of the ITS-SSU sequence as *N. ceranae* does not belong to the 'true *Nosema*' group (characterized by the reversed rRNA gene arrangement). *Encephalitozoon* species are not an appropriate outgroup for analysis of the ITS-SSU sequence since the identical non-coding region next to the 5' end of SSU had extremely low similarity. However, as the phylogenetic analysis of RPB1 concerns only one gene region, the arrangement of genes did not affect our selection of outgroups and the RPB1 gene of *N. ceranae* is too similar for this species to be used as an outgroup in this analysis. Thus, two *Encephalitozoon* species were used as the outgroup in the phylogenetic analysis of RPB1 because these are well studied species and are known to be distinct from *Nosema* species (Vossbrinck and Debrunner-Vossbrinck, 2005). The computed output was visualized using MEGA version 6.0 (Tamura et al., 2013).

Statistical analysis

Statistical analyses were carried out using R version 3.1.2 (R Core Team, 2014). A t-test and a Wilcoxon signed-rank test were used to compare the dimensions of fresh mature spores and the number of polar filament coils from the TEM images. All other analyses used generalized linear models (GLMs). Treatment (healthy or infected) and gender (male or female) were used as explanatory variables for analyzing each of the larval and pupal life history performance measurements, while treatment was the only explanatory variable for analyzing the measurements of adult female performance. As gender identity was assessed at pupation, gender could be used in analyzing larval development time, but could not be applied to larval survivorship. Analysis of daily fecundity, and egg hatch was confined to those females that successfully oviposited. Full models that included explanatory variables and two-way interactions were used initially, and stepwise model simplification was performed manually using likelihood-ratio tests (χ^2) in the absence of overdispersion and *F* tests to incorporate an empirical scale parameter in the presence of overdispersion (Crawley, 2013). Only significant interactions are presented in the results. Standard link functions were used for the GLMs and error distributions were selected to best represent the measurement variables analyzed (Gaussian for continuous variables, Poisson for counts, binomial for proportions). We ensured that error distributions were appropriate by inspecting plots of residuals or standardized deviance versus predicted values, and normal quantile plots (Crawley, 2013).

2.4 RESULTS

Pathology and vertical transmission

All statistical analyses of life table measurements from *E. postivittana* larvae that were either uninfected (healthy) or infected with *N. fumiferanae postvittana* are presented in Table 1. Larval

development time (days) was longer for infected females and males (21.50 ± 0.66 SE, 19.32 ± 0.58 SE respectively) compared to healthy females and males (20.33 ± 0.40 SE, 17.27 ± 0.30 SE respectively). Larval and pupal survivorships were lower for infected individuals compared to healthy individuals (Fig. 1A & B). Pupal fresh weight (mg) was greater for infected females and males (52.43 ± 1.52 SE, 30.74 ± 0.54 SE) compared to healthy females and males (49.56 ± 0.91 SE, 28.55 ± 0.53 SE). Pupal development time was slightly shorter for females (9.84 ± 0.15 SE) compared to males (10.83 ± 0.13 SE), but did not differ significantly between infected and healthy treatments. The percentage of females did not differ significantly between infected (43 ± 5 SE) and healthy (47 ± 5 SE) treatments. A significantly lower proportion of infected females eclosed (73 ± 8% SE) oviposited compared to the healthy females (93 ± 4% SE). Infected females also had reduced longevity, daily fecundity, and percent egg hatch compared to healthy females (Figs. 2A-C).

From the 17 infected *E. postvittana* females that survived to reproduce, 67 ± 6% (SE, $n = 68$) of the dissected egg masses collected on days 2, 6 and 10 showed visible signs of infection (spores were present) and thus confirmed vertical transmission. However, as we did not surface sterilize the eggs before dissection it was not possible to determine whether the presence of spores was due to transovarial transmission at this stage. More than 10^7 spores were counted in each of the ovipositing females at time of death ($2.2 \times 10^8 \pm 3.3 \times 10^7$ SE). There was no effect of female age (2, 6, or 10 days) (GLM (binomial), $\chi 2 = 3.51$, $df = 2$, $P = 0.17$), female spore load (GLM (binomial), $\chi 2 = 0.64$, $df = 1$, $P = 0.64$), or egg mass size (GLM (binomial), $\chi 2 = 0.06$, $df = 1$, $P = 0.81$) on the prevalence of infection in egg masses. Of the small number of first instar larvae that hatched from eggs collected on days 2, 6 and 10, mature spores were present in 100 ± 0% (SE, $n = 12$), confirming transovarial transmission.

Tissues infected and morphology

Observations of inoculated fifth instar *E. postvittana* larvae revealed that infection began in the midgut. Spores were observed in the midgut, Malphigian tubules and the silk glands (3 dpi), followed by the hemolymph (8 dpi), the fat body (8 dpi), and finally the developing gonads (10 dpi). All major organs were infected with spores at 10 dpi. Mature spores of both *N. fumiferanae* isolates were elongate ellipsoidal and highly refractive under phase contrast. Fresh mature spores of *N. fumiferanae postvittana* (Fig. 3A) measured 3.9 (± 0.1 SE) x 1.8 (± 0.1 SE) μm and those of *N. fumiferanae* measured 3.8 (± 0.1 SE) x 1.9 (± 0.0 SE) μm ($n = 10$ for each isolate) and did not differ significantly between isolates (t-test, $t_{length} = 0.55$, $P = 0.59$; $t_{width} = 0.79$, $P = 0.44$).

Images of the life cycle stages and ultrastructure of the two microsporidian isolates, *N. fumiferanae postvittana* and *N. fumiferanae* are presented in Fig. 4 and Fig. 5 respectively. All stages were diplokaryotic and from the second sporulation cycle. *N. fumiferanae postvittana* had significantly more polar filament coils (Wilcoxon signed-rank test, $V = 78.5$, $n = 41$, $P = 0.001$) arranged in a row (range 12-15, mean 14.32 ± 0.20 SE, $n = 20$, Fig. 4D, E) than *N. fumiferanae* (range 11-15, mean 12.86 ± 0.30 SE, $n = 21$, Fig. 5D, E). Polar filament coils were isofilar and arranged in single rows. The mature spores of both isolates possess a horseshoe shaped anchoring disk (relatively long arms), a lamellar polaroplast (Fig. 4D, E and Fig. 5D, E), and thick endospore. The meronts (Fig. 4A, 5A) were nested in the cytoplasm of the host cell, and were limited by a plasmalemma, which thickened at the sporont stage (Fig. 4B, 5B). The

cytoplasm of the meronts, sporonts and sporoblasts contained ribosomes, cisternae of the rough and smooth endoplasmic reticulum and Golgi vesicles. Free ribosomes appeared in the meront and sporont stages, but were bound in the sporoblast and mature spores (Fig. 4, 5).

Sequence comparison and phylogenetic analysis

We amplified a 110 base pair (bp) fragment of ITS, a 1178 bp fragment of SSU and a 562 bp fragment of RPB1 from purified *N. fumiferanae postvittana* spores isolated from three host individuals of *E. postvittana*. The fragment sequences of ITS and SSU we obtained were identical from the three host individuals and were highly similar to published sequences for *N. fumiferanae,* with only 6 bp differences observed between the isolates for both loci (two insertions and one polymorphic site for SSU, and three polymorphic sites for ITS). Sequencing of the forward primer of RPB1 failed, so our results are based on the sequences from the reverse primer only. No notable polymorphisms were found within sequences from RPB1, and *N. fumiferanae postvittana* shared 97% sequence similarity (12 bp differences) to published sequences from *N. fumiferanae*. These 12 bp differences corresponded to 10 synonymous and 2 non-synonymous mutations.

Phylogenetic reconstructions for ITS-SSU and RPB1 are presented in Figures 6 and 7, respectively. Our reconstruction for ITS-SSU only included sequences from *Nosema* species, but we identified three well-supported clades (> 91 bootstrap probability [BP]), with *N. ceranae* as the outgroup. One clade included published sequences for *N. philosamiae* and *N. antheraeae* (92 BP), a second clade included the sequence from *N. fumiferanae postvittana*, the published sequence for *N. fumiferanae,* and sequences from two unidentified *Nosema* species isolated from other *Choristoneura* hosts (93 BP), and the third clade included sequences from *N. bombycis, N. heliothidis, N. spodopterae*, and three sequences from unidentified *Nosema* spp. (91 BP). In general, the results from the reconstruction of RPB1 showed strong phylogenetic structure with several well-supported clades and outgroup (*Encephalitozoon* spp. [100 BP]). One clade (78 BP) included published sequences for *N. granulosis* and *V. cheracis,* another clade (100 BP) included sequences from *N. apis, N. ceranae*, *V. disparis,* and *V. necatrix*, and a third clade (99 BP) included the sequence from *N. fumiferanae postvittana, N. fumiferanae,* as well as published sequences from *N. antheraeae, N. bombycis*, *N. tyriae* and two unidentified *Nosema* species isolated from other *Choristoneura* hosts. Phylogenetic reconstructions for both loci confirm the relatedness of *N. fumiferanae postvittana* to *N. fumiferanae* (Table S2.2).

2.5 DISCUSSION

Spores of *N. fumiferanae postvittana* were present in all tissues of fifth instar larval hosts 10 days post inoculation with 10^3 spores. Larval mortality was 7% higher for individuals infected as second instar larvae and mortality was 13% higher in infected pupae compared to healthy individuals. Furthermore, the pupation period was two days longer for infected individuals than for healthy individuals. Although males developed faster than females, we did not find a differential effect of gender on mortality. Adult females that were inoculated as second instar larvae died five days earlier, produced 47% fewer eggs in their lifetime, and experienced 22% lower egg hatch than healthy females. The majority of egg masses (68%) and all hatched larvae produced by infected females confirmed vertical transmission. These observations of systemic

tissue infection, moderate pathology and transmission of *N. fumiferanae postvittana* from infected adults to their offspring, support the strategy of a low virulence pathogen that develops slowly, allows its host to continue development to the adult stage and is maintained in the host population by vertical transmission (Anderson and May, 1981; Solter, 2006).

The pathology and transmission of *N. fumiferanae postvittana* with respect to *E. postvittana* is similar to that reported for *N. fumiferanae* for *C. fumiferana* (Thomson, 1957; Bauer and Nordin, 1989). Vertical transmission in *C. fumiferana* was also found to result in 100% infection of hatching larvae in a study by Bauer and Nordin (1989). However, van Frankenhuyzen et al. (2007) found vertical transmission to be dependent on infection intensity in the parent female, with 100% transmission to eggs and larvae occurring only when females contained more than 4.5-5 x 10^5 spores. Although we found variation in the extent of vertical transmission among parent females, we did not find a relationship with infection intensity. However, in our study, all ovipositing females contained over 10^7 spores at the time of death.

One difference in the pathology of the *Nosema* isolates from *E. postvittana* and *C. fumiferana* appears to be the effect of infection on host pupal fresh weight. Thomson (1957) and Bauer and Nordin (1989) found that infected *C. fumiferana* pupae were significantly smaller than healthy pupae. Reduced pupal fresh weight was consistent for larvae infected via vertical transmission and for larvae infected as fourth and fifth instars (Bauer and Nordin, 1989). These observations contrast with our results from *E. postvittana* larvae infected as second instars, for which pupal fresh weight was greater than that of healthy individuals for both males and females. We did not measure the dimensions of the *E. postvittana* pupae, so it remains unknown whether the increased fresh weight of infected pupae was due to additional growth of the host larvae (which required a slightly longer time period to complete their development), or to induced cell hypertrophy (Vavra and Lukes, 2013).

In addition to the similarities in host pathology between *N. fumiferanae* and *N. fumiferanae postvittana*, both isolates also exhibit the highly conserved reversed arrangement of rRNA gene subunits, in which the sequence order is LSU-ITS-SSU (Huang et al., 2004). This further supports the suggestion that the genus *Nosema* should be reserved for species that have the reversed arrangement of rRNA gene subunits (Huang et al., 2004; Tsai et al., 2005; Kyei-Poku et al., 2008; Kyei-Poku et al., 2012). The SSU fragment is very similar among *Nosema* species, with 98-100% similarity among species in the *N. bombycis* group (Tsai et al., 2005; Kyei-Poku et al., 2008). We found only three nucleotide differences in the sequences from *N. fumiferanae postvittana*, *N. fumiferanae*, *N. bombycis*, and *Nosema* sp. CPP (isolated from *Choristoneura pinus pinus* Freeman), and eight nucleotide differences from *Nosema* sp. CO (isolated from *Choristoneura occidentalis* Freeman). Similar differences in SSU sequences have been found for other *Nosema* species that have non-overlapping host ranges (Tsai et al., 2005).

In both of our phylogenetic reconstructions (ITS-SSU and RPB1), we found that our isolate, *N. fumiferanae postvittana,* was placed within the *N. bombycis* group and within the *N. fumiferanae* species complex along with two other closely related isolates (*Nosema* sp. CPP and *Nosema* sp. CO) from *Choristoneura* hosts (Kyei-Poku et al., 2008). In general, we also found that the topologies of both reconstructions were broadly congruent in that the *N. bombycis* group formed a distinct clade in both analyses, though this clade was not well supported in either

reconstruction. One difference between the reconstructed topologies was the placement of *N. antheraeae* which may have been a result of the relatively short branch lengths observed between individuals in the ITS-SSU analysis (as suggested by the lack of support), the independent evolutionary histories of these two loci, and/or because these sequences were obtained from GenBank and may be products of species-level misidentification (Vilgalys, 2003). Despite these differences, our results demonstrate overall congruence between the reconstructions from these two markers and add further support to the growing use of RPB1 for both species-level and higher taxonomic analyses (Hirt et al., 1999; Cheney et al., 2001; Vavra et al., 2006a; Gisder and Genersch, 2013; Luo et al., 2014).

According to our morphological and phylogenetic analyses, we consider the isolate, *N. fumiferanae postvittana*, to be part of the *Nosema fumiferanae* species complex, along with *Nosema* sp. CO and *Nosema* sp. CPP. Thus, *N. fumiferanae postvittana* is potentially a Nearctic species originating from resident tortricid hosts, and shares a very close evolutionary relationship with other members of the *N. fumiferanae* species complex isolated from North America forest habitats. Nevertheless, we cannot exclude the possibility that *N. fumiferanae postvittana* was carried by *E. postvittana* (also in the family Tortricidae) when it invaded California, as no studies of microsporidian infection of this host in Australia have been published.

The similarity of the ultrastructure images of both *N. fumiferanae* isolates further supports their relatedness, and their separation from *N. bombycis*. The ultrastructure images of *N. bombycis* (Sato et al., 1982) demonstrate a bulbous-shaped anchoring disk compared to the horseshoe shaped anchoring disk in the two *N. fumiferanae* isolates. The two rows of polar filament coils are also more symmetrical in the two *N. fumiferanae* isolates than in *N. bombycis*. However, the mean number of polar filament coils in mature spores of *N. bombycis* was observed to be 12.21 (Sato et al., 1982), very similar to the 12.86 coils that we observed in spores of *N. fumiferanae*, but fewer than the 14.32 coils that we observed in spores of *N. fumiferanae postvittana* and the 14.71 coils observed for *Nosema* sp. M12 (Sato et al., 1982). It should be noted, however, that the polar filament coil counts were made from spores of *N. fumiferanae* infecting *E. postvittana* rather than *C. fumiferana,* the natural host of the isolate.

While we did not specifically test the host range of *N. fumiferanae postvittana.,* we discovered that an egg parasitoid, *Trichogramma fasciatum* (Perk.) and a larval endoparasitoid, *Meteorus ictericus* Nees, were also susceptible to infection when reared in infected *E. postvittana* (Hopper, unpublished observations). This was not surprising as infections by microsporidia in parasitoids of infected hosts are not uncommon (Bjornson and Oi, 2014) and spores of *N. fumiferanae* were recovered from two larval parasitoids of *C. fumiferana*: *Apanteles fumiferanae* and *Glypta fumiferanae* (Thomson, 1958a).

If the *N. fumiferanae* species complex is North American in origin, the acquisition of an indigenous microsporidium by *E. postvittana* may have occurred via horizontal transmission from a resident insect host (possibly a tortricid) or the intervention of a vector, such as parasitoids or birds. The ability of parasitoids to vector microsporidia to uninfected hosts is variable (Own and Brooks, 1986; Siegel et al., 1986; Hoch et al., 2000; Futerman et al., 2006; Simões et al., 2012; Saito and Bjornson, 2013), but birds can act as potential vectors by

dispersing pathogens after consuming infected prey (Entwistle et al., 1978; Slodkowicz-Kowalska et al., 2006).

In conclusion, from the pathology, morphology and sequence results, we cannot determine with certainty whether *N. fumiferanae postvittana* is a novel species. We suggest that it belongs to the *N. fumiferana* species complex that also includes *Nosema* sp. CO and *Nosema* sp. CPP. Although we cannot determine the origin of this isolate without further exploration of the host in its native range, this novel pathogen potentially contributes further to the biotic resistance that *E. postvittana* has experienced from other resident natural enemies in California (Wang et al., 2012; Bürgi and Mills, 2014; Bürgi et al., 2015).

2.6 TABLE

Table 1. Statistical analysis of the effect of infection by *Nosema fumiferanae postvittana* on the life history performance of *Epiphyas postvittana*.

Model	*N*[a] (H, I)	GLM family, statistic	*df*	*P*
Larval survivorship	101, 95	binomial, $\chi 2$		
Treatment		4.71	1	0.03
Larval development time	98, 85	Poisson, $\chi 2$		
Treatment		6.36	1	0.01
Gender		16.38	1	<0.001
Pupal sex-ratio	98, 85	binomial, $\chi 2$		
Treatment		0.09	1	0.76
Pupal fresh weight (g)	98, 85	gaussian, F		
Treatment		8.13	1, 180	0.01
Gender		586.75	1, 180	< 0.001
Pupal survivorship	98, 85	binomial, $\chi 2$		
Treatment		13.45	1	< 0.001
Gender		1.24	1	0.27
Pupal development time	97, 73	Poisson, $\chi 2$		
Treatment		2.67	1	0.10
Gender		4.01	1	0.05
Female longevity	46, 33	Poisson, $\chi 2$		
Treatment		29.26	1	<0.001
Oviposition	46, 33	binomial, $\chi 2$		
Treatment		6.45	1	0.01
Daily fecundity	46, 33	Poisson, $\chi 2$		
Treatment		41.88	1	<0.001
Egg hatch	43, 23	binomial, $\chi 2$		
Treatment		1124.1	1	<0.001

[a] Number of healthy (H) and infected (I) individuals.

Table S2.1. Primers and PCR conditions used for amplification and sequencing of fragments of three commonly used loci from *N. fumiferanae postvittana*.

Marker	Primer[a]	Sequence 5'-3'	Annealing °C	Elongation time
ITS	ILSUF	ACT CTC CTC TTT GCC TCA ATC A	50	45s
	530r	CCG CGG KGC TGG CAC	50	
SSU	18f	CAC CAG GTT GAT TCT GCC	51	90s
	1537r	TTA TGA TCC TGC TAA TGG TTC	51	
RPB1	Rpb1-f1	CGG ACT TYG AYG GNG AYG ARA TGA	50	70s
	Rpb1-r1	CCC GCK NCC NCC CAT NGC RTG RAA	50	

[a]Primers 530r, 18f, 1537r are from Vossbrinck et al. (1993). ILSUF (Tsai et al., 2002) is the complementary sequence to ILSUR. Rpb1f1 and Rpb1r1 are from Hirt et al. (1999).

Table S2.2. Estimates of base composition bias differences, RPB1 and ITS-SSU sequences. RPB1 sequences, 495 positions (including gaps), are in the top-right of the table (bold), and ITS-SSU sequences, 1345 positions, are in the bottom-left. RPB1 sequences exhibited higher base composition bias differences within the true *Nosema* group (1-6). *N. ceranae* exhibited distinct differences The pairwise comparisons were conducted using MEGA vs. 6.0.

	1	2	3	4	5	6	7
1. *N. fumiferanae postvittana*		**0.023**	**0.022**	**0.020**	**0.070**	**0.081**	**0.353**
2. *N. fumiferanae*	0.004		**0.025**	**0.025**	**0.072**	**0.077**	**0.352**
3. *Nosema* sp. CO	0.005	0.002		**0.025**	**0.069**	**0.079**	**0.356**
4. *Nosema* sp. CPP	0.005	0.010	0.016		**0.078**	**0.083**	**0.360**
5. *N. bombycis*	0.002	0.002	0.005	0.009		**0.059**	**0.368**
6. *N. antheraeae*	0.019	0.040	0.033	0.021	0.032		**0.359**
7. *N. ceranae*	0.699	0.664	0.705	0.602	0.663	0.795	

Table S2.3. DNA fragments used in the phylogenetic analyses

GenBank #	**ITS-SSU fragment**	**Host**
AY747307	*Nosema spodopterae*	*Spodoptera litura*
JN882299	*Nosema* sp. HA	*Hemerophila atrilineata*
FJ772435	*Nosema heliothidis*	*Helicoverpa armigera*
AY960986	*Nosema* sp. PX1	*Plutella xylostella*
AY259631	*Nosema bombycis*	*Bombyx mori*
FJ969508	*Nosema* sp. PA	*Phyllobrotica armata*
KT020736	***Nosema fumiferanae postvittana* subsp. n.**	***Epiphyas postvittana***
HQ457434	*Nosema* sp. CO	*Choristoneura occidentalis*
HQ457432	*Nosema fumiferanae*	*Choristoneura fumiferana*
HQ457433	*Nosema* sp. CPP	*Choristoneura pinus pinus*
HQ457431	*Nosema disstriae*	*Malacosoma disstria*
DQ073396	*Nosema antheraeae*	*Antheraea pernyi*
FJ767862	*Nosema philosamiae*	*Samia cynthia ricini*
DQ486027.1	*Nosema ceranae*	*Apis mellifera*

GenBank #	**RPB1 partial gene**	**Host**
DQ996231	*Nosema bombycis*	*Bombyx mori*
JX213753	*Nosema bombycis* isolate Bm9	*Bombyx mori*
AJ278948	*Nosema tyriae*	*Tyria jacobaeae*
HQ215550	*Nosema antheraeae*	*Antheraea pernyi*
HQ457435	*Nosema fumiferanae*	*Choristoneura fumiferana*
HQ457436	*Nosema* sp. CO	*Choristoneura occidentalis*
KT020735	***Nosema fumiferanae postvittana* subsp. n.**	***Epiphyas postvittana***
HQ457437	*Nosema* sp. CPP	*Choristoneura pinus pinus*
DQ996230	*Nosema apis*	*Apis mellifera*
JX239748	*Vairimorpha disparis*	*Lymantria dispar*
DQ996236	*Vairimorpha necatrix*	*Pseudaletia unipuncta*
XM_002995356	*Nosema ceranae*	*Apis mellifera*
DQ996233	*Nosema granulosis*	*Gammarus duebeni*
DQ996235	*Vairimorpha cheracis*	*Cherax destructor*
KC513611	*Encephalitozoon cuniculi*	*Mus musculus*
XM_003886848	*Encephalitozoon hellem*	*Homo sapiens*

2.7 FIGURES

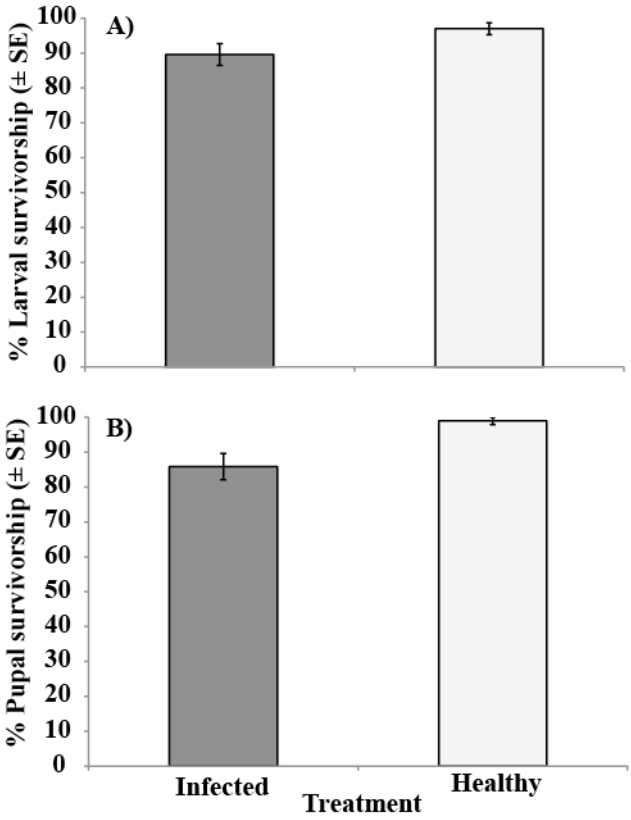

Figure 1. Mean (± SE) larval survivorship (A) and pupal survivorship (B) for *Epiphyas postvittana* infected by *Nosema fumiferanae postvittana*, compared to healthy individuals.

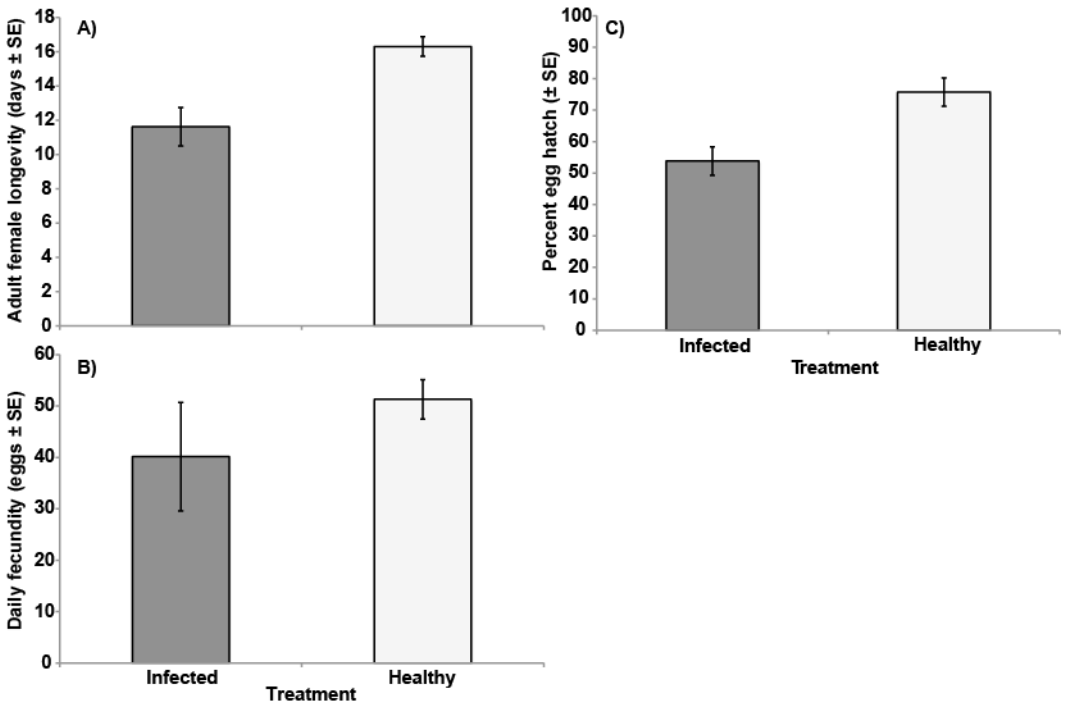

Figure 2. Mean (± SE) adult longevity (A), daily fecundity (B), and the percent egg hatch (C) for *Epiphyas postvittana* infected by *Nosema fumiferanae postvittana*, compared to healthy individuals.

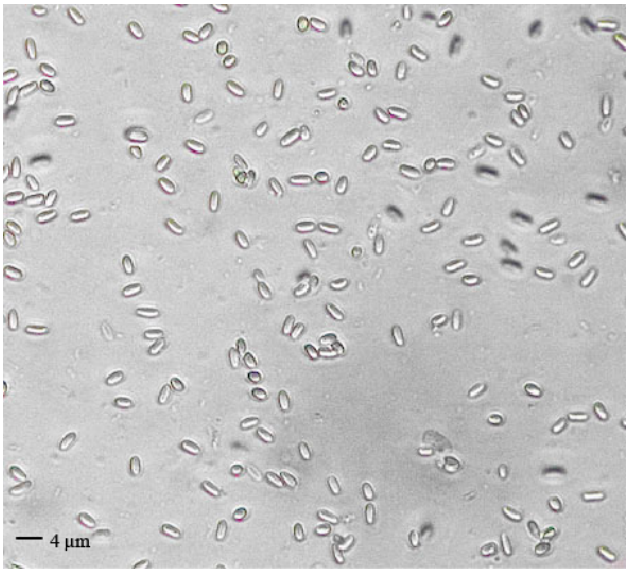

Figure 3. Light microscopy of *N. fumiferanae postvittana* spores at 400x with phase contrast.

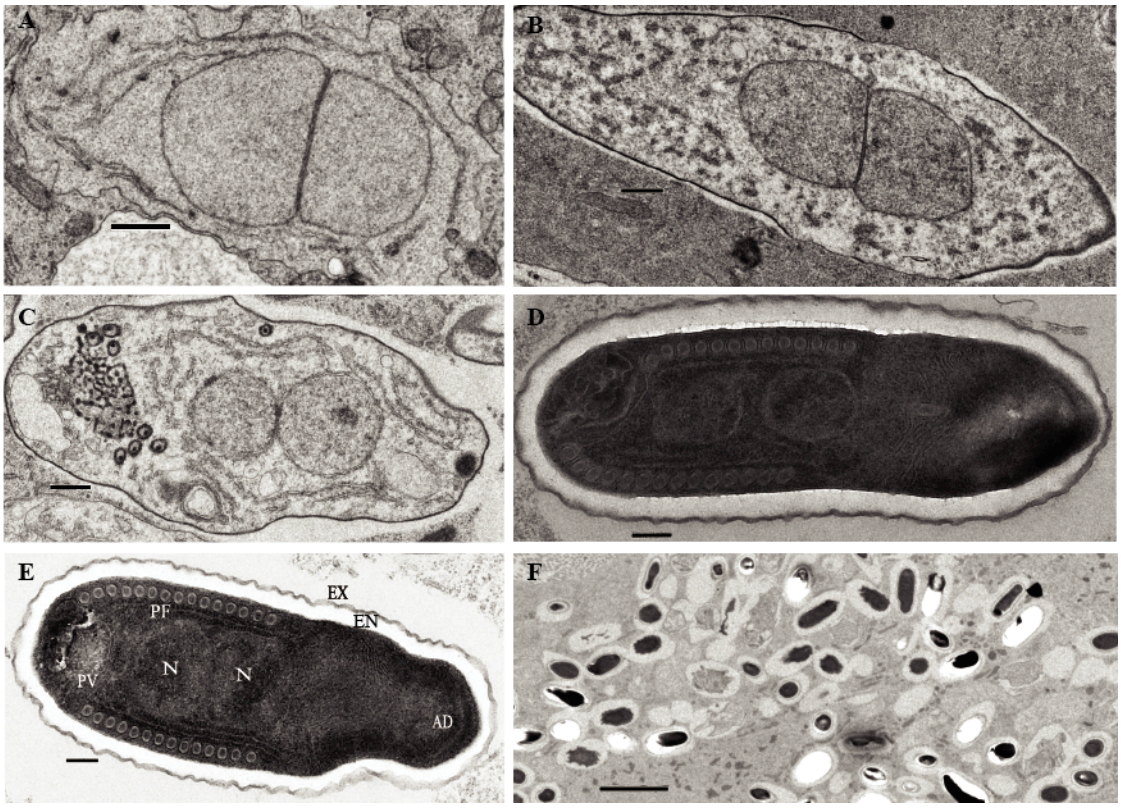

Figure 4. Transmission electron micrographs of the developmental stages of *Nosema fumiferanae postvittana* in the midgut of the host, *Epiphyas postvittana*. (A) Binucleate meront, bar = 0.5 μm. (B) Sporont, bar = 0.5 μm. (C) Sporoblast; bar = 0.5 μm. (D) Mature spore, bar = 0.2 μm. (E) Mature spore, bar = 0.2 μm; AD = anchoring disk; = exospore; EN = endospore; N = nucleus; PF = polar filament; PV = polar vacuole. (F) All developmental stages; bar = 5 μm.

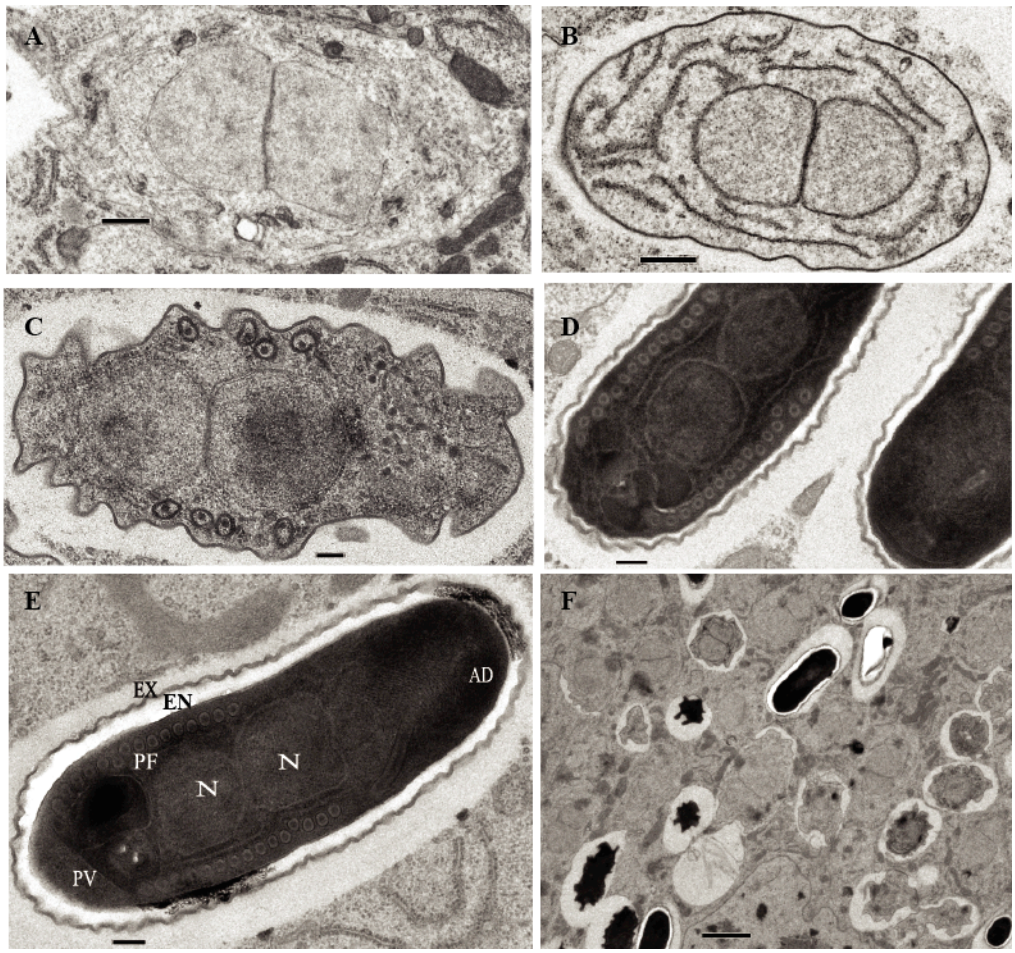

Figure 5. Transmission electron micrographs of the developmental stages of *Nosema fumiferanae* in the midgut of the host, *Epiphyas postvittana*. (A) Binucleate meront; bar = 0.5 μm. (B) Sporont; bar = 0.5 μm. (C) Sporoblast; bar = 0.2 μm. (D) Mature spore, bar = 0.2 μm. (E) Mature spore, bar = 0.2 μmm; AD = anchoring disk; = exospore; EN = endospore; N = nucleus; PF = polar filament; PV = polar vacuole. (F) All developmental stages; bar = 2 μm.

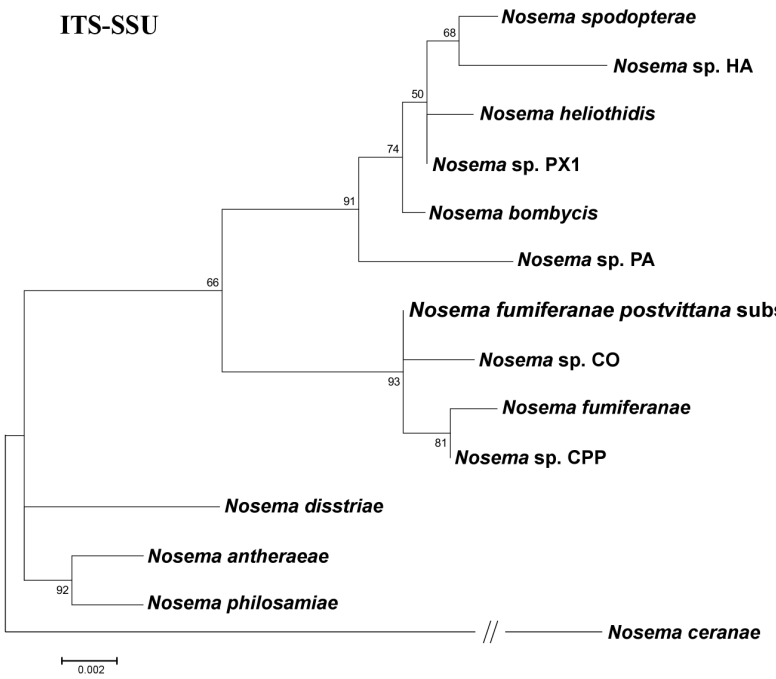

Figure 6. Phylogenetic tree of non-polymorphic ITS-SSU gene nucleotides. The numbers are the supporting values of the nodes from 1000 bootstrap replicates. *Nosema ceranae* was used as the outgroup for rooting the ITS-SSU tree (with a shortened branch length to fit the figure). GenBank accession numbers are in Table S2.3.

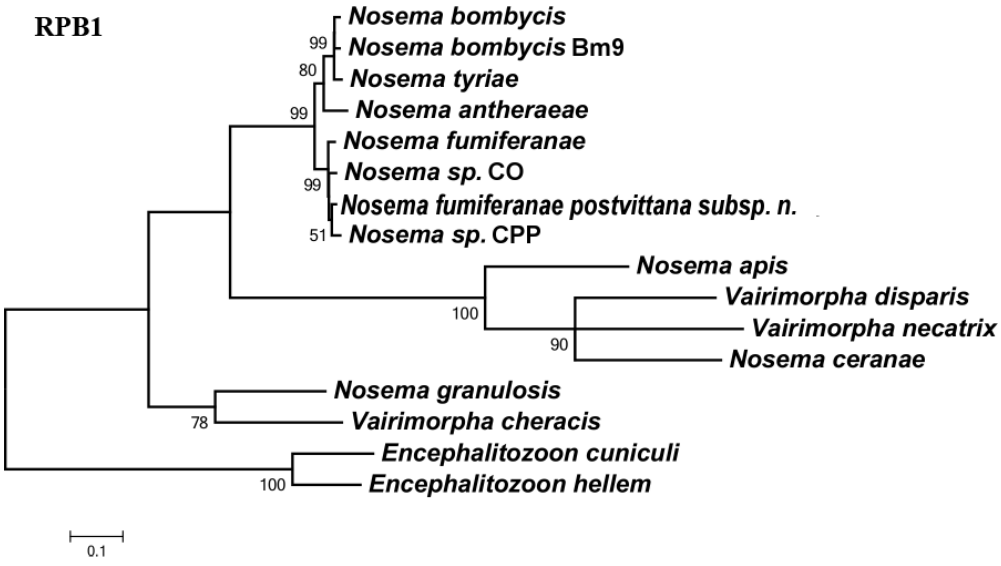

Figure 7. Phylogenetic tree of RPB1 gene nucleotides. The numbers are the supporting values of the nodes from 1000 bootstrap replicates. *Encephalitozoon* species were used as the outgroup for rooting the RPB1 tree. GenBank accession numbers are in Table S2.3.

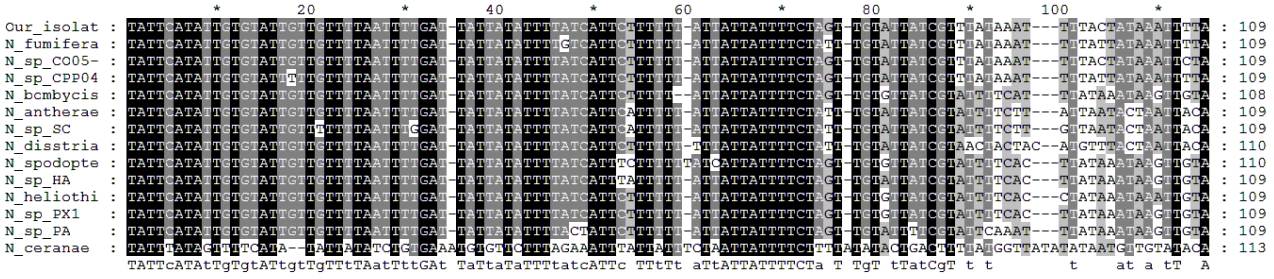

Figure S2.1. Non-polymorphic ITS region alignment, the 5' poorly aligned region (one base pair) and SSU were trimmed. The alignment with SSU was used in the ITS-SSU phylogenetic analysis.

CHAPTER 3

PATHOGENICITY, PREVALENCE AND INTENSITY OF A MICROSPORIDIAN INFECTION BY *NOSEMA FUMIFERANAE POSTVITTANA* IN THE LIGHT BROWN APPLE MOTH, *EPIPHYAS POSTVITTANA*, IN CALIFORNIA

3.1 ABSTRACT

While biological invasions are increasing, the post-establishment fates of exotic species are varied. In some cases, these newly arrived species exhibit an initial phase of population growth and spread, followed by a subsequent phase of natural decline. The light brown apple moth, *Epiphyas postvittana* (Walker) (Lepidoptera: Tortricidae), provides a unique opportunity to examine potential mechanisms for the natural suppression of an exotic insect species that has become established in coastal California. We recently discovered a microsporidian pathogen, *Nosema fumiferanae postvittana*, from *E. postvittana* in its novel range. In the laboratory, we examined the pathogenicity and latent period of this microsporidium, and in the field we determined its prevalence and intensity in five locations using quantitative real-time PCR (qPCR). In the laboratory, when comparing healthy larvae to larvae infected with up to 10^5 spores, we found a reduction in juvenile survivorship (from 100% to 26%), a prolongation of juvenile development time (of up to 9-10 days), a reduction in viable lifetime fecundity (from 788 to 1) and a reduction in the intrinsic rate of increase (from 0.18 to 0.008). The median lethal dose (LD_{50}) was estimated to be 1.8×10^4 spores, and the mean latent period for infections with 10^3 spores was 12.67 days. Our field sampling revealed that *E. postvittana* populations have further declined from previously reported densities in San Francisco and Santa Cruz. We detected *N. fumiferanae postvittana* in all five locations with an overall prevalence of 5%, which did not appear to be influenced consistently by either host density or season. Mean microsporidian intensity in field-infected individuals was 226 spores. Although the laboratory results demonstrated the potential for host suppression, the field sampling indicated that the prevalence and intensity of microsporidian infection were too low to account for the continued decline in population densities of *E. postvittana* in coastal California.

3.2 INTRODUCTION

Biotic invasions are recognized as being one of the main drivers of global environmental change (Sala et al., 2000; Tylianakis et al., 2008). Invasive species can disrupt and modify invaded communities through direct and indirect interactions and cause both ecological and economic repercussions (Mack et al., 2000; Ricciardi et al., 2013). The success of an invasive species is dependent on a variety of abiotic and biotic factors, such as propagule pressure, climate compatibility, resource abundance, adaptive traits, and the presence of resident competitors and natural enemies (Strayer et al., 2006; Davis, 2009; Simberloff, 2009; Ricciardi et al., 2013). The majority of invasive species escape from their coevolved natural enemies (Keane and Crawley, 2002; Mitchell and Power, 2003; Torchin et al., 2003), but in some cases can either act as a transport vector for coevolved parasites (Strauss et al., 2012), or encounter biotic resistance from novel resident competitors or enemies in a new region (Elton, 1958; Maron and Vila, 2001; Levine et al., 2004; Carlsson et al., 2011; Dumont et al., 2011; Alofs and Jackson, 2014).

Whether biotic resistance can limit the population growth and spread of an invasive species often depends on spatial and temporal synchronization as well as the strength of interactions with resident natural enemies and competitors (e.g. see Maron and Vila, 2001; Alofs and Jackson, 2014). While many of the exotic species that establish in new geographic regions fail to become truly invasive, the extent to which biotic resistance may be responsible remains largely unknown as such populations are both more difficult to detect and to study (Zenni and Nunez, 2013). In other cases, however, exotic species exhibit an initial phase of population growth and spread, followed by a subsequent phase of natural decline (Simberloff and Gibbons, 2004; Zenni and Nunez, 2013). Such species provide great opportunities to study the factors that can contribute to the barrier between establishment and spread for potential invasive species (Zenni and Nunez, 2013).

The light brown apple moth, *Epiphyas postvittana* (Walker) (Lepidoptera: Tortricidae), provides a unique opportunity to examine potential mechanisms for the natural suppression of an exotic insect species that has become established in coastal California. Native to Australia, *E. postvittana* is a leafroller that was confirmed to be present in California in 2006 (Brown, 2007), and initially increased in abundance at an exponential rate in four counties that were monitored extensively from 2007 to 2010 (Suckling et al., 2014). More recently, however, populations of *E. postvittana* in Santa Cruz and San Francisco, the initial focal points of establishment, were observed to be in decline (Bürgi et al., 2015). *E. postvittana* is highly polyphagous, feeding on over 500 host plant species (Brockerhoff et al., 2011), and therefore has the potential to interact both directly and indirectly with many resident species in the invaded region.

Since its accidental introduction to California, *E. postvittana* has attracted the attention of a wide range of resident parasitoids (Wang et al., 2012; Bürgi and Mills, 2014; Bürgi et al., 2015) and generalist predators (Hogg et al., 2013). Bürgi et al. (2015) investigated the effect of parasitism by resident parasitoids on the dynamics of *E. postvittana* populations in San Francisco and Santa Cruz over a four-year period. Although they document substantial levels of parasitism, they found no evidence that parasitism influenced per capita population growth rates of their newly adopted host. In contrast, they found evidence of strong compensatory feedback [a decrease in

per capita growth rate with increasing population size (Herrando-Perez et al., 2012)] due to unknown factors. While factors contributing to population declines of naturalized exotic species are often poorly understood, pathogens have occasionally been considered to be at least partially responsible (Simberloff and Gibbons, 2004).

The influence of pathogens on insect populations has often been overlooked in comparison to that of parasitoids and predators, as they are more difficult to detect and their effects are not as easily quantified (Anderson and May, 1981; Solter, 2014). Recently, we discovered that larvae of *E. postvittana* in California were infected with a microsporidian pathogen, *Nosema fumiferanae postvittana* (Chapter 2). We determined that this isolate belongs to the *N. fumiferanae* species complex, that it is both horizontally and vertically transmitted, and that it has negative effects on the fitness of its novel host.

Vertical transmission facilitates the persistence of pathogens at low host densities, while traits such as persistence, infectivity and pathogenicity determine their potential to suppress host populations at higher densities (Anderson and May, 1981; Briggs and Godfray, 1995). For Microsporidia, however, a high level of infectivity and/or pathogenicity is not always associated with a greater impact on host populations (Solter, 2014) as asymptomatic infections and subtle sublethal effects can also have profound impacts (Bonsall et al., 2005; Solter et al., 2012a; Solter, 2014). In this context, several studies have noted the importance of microsporidian infection in insect hosts (Thomson, 1958b; Hill and Gary, 1979; Wilson, 1987; Lewis et al., 2009), and seven different species of Microsporidia have been deliberately introduced for the biological control of insect pests (Hajek and Delalibera, 2010). Microsporidia can act in concert with other factors in the suppression of insect populations (Lewis et al., 2009; van Frankenhuyzen et al., 2011; Solter, 2014), but in some cases, may also be negatively influenced by competing natural enemies (Eveleigh et al., 2012).

To understand the significance of the novel pathogen-host relationship between *N. fumiferanae postvittana* and *E. postvittana,* we conducted laboratory experiments and field sampling. The objectives of the laboratory experiments were to 1) estimate the median lethal dose (LD_{50}) and the effect of dose ingested on the life-history performance of *E. postvittana* larvae as key aspects of the pathogenicity of *N. fumiferanae postvittana*, and 2) estimate the latent period (time from initial host infection to production of infectious spores), an essential parameter for understanding disease dynamics (Anderson and May, 1981; Briggs and Godfray, 1995; Goertz and Hoch, 2011; Solter, 2014). We expected the LD_{50} of *N. fumiferanae postvittana* to be greater than or equal to that determined for *Choristoneura fumiferana* (Clem.) (Tortricidae) larvae infected with *N. fumiferanae* (2.23×10^4 spores), and for sublethal effects to increase with increasing spore dose (Bauer and Nordin, 1988). We also expected that the latent period for our isolate would be comparable to the 15 days found for *N. fumiferanae* in *Choristoneura occidentalis* (Walsingham) (Campbell et al., 2007) and for *Nosema lymantriae* in *Lymantria dispar* L. (Lymantriidae) (Goertz and Hoch, 2008).

The objective of the field sampling was to determine the prevalence (percentage of hosts infected at a particular point in time) and intensity (spore load of an infected host at a particular point in time) of infection (Bush et al., 1997) by *N. fumiferanae postvittana* in five field populations of *E. postvittana* in coastal California using quantitative real-time PCR (qPCR). We used qPCR as a

tool for pathogen detection and quantification as low intensity infections are easily overlooked through light microscopy (Refardt and Ebert, 2006). We expected pathogen prevalence and intensity to be dependent on host density with a seasonal lag time, as observed for other Microsporidia (Kohler and Hoiland, 2001; Lewis et al., 2009), and to vary among plant species due to differences in nutritional quality and plant defenses (Cory and Hoover, 2006).

3.3 MATERIALS AND METHODS

Life history consequences of spore dose

Spores of *N. fumiferanae postvittana* were isolated in 2014 from a laboratory colony of *E. postvittana* that was initially established from larvae collected in Santa Cruz, California in 2007 and supplemented with adult females collected from Richmond and Berkeley, California in 2013. Infected and healthy host colonies were maintained at 20°C, a 16:8 h L:D photoperiod, and a relative humidity above 60%, and all experiments were conducted under the same conditions. The healthy colony was located in an insectary and separate growth chambers were used for the infected colony and for all experiments. We used 10% bleach to sterilize all tools, containers and surfaces prior to use. An uninfected laboratory colony of *E. postvittana* was established from egg masses donated by USDA-APHIS from a separate colony of the same original source population from Santa Cruz, California. Larvae were reared on an artificial bean-based diet developed by Cunningham (2007). Upon pupation, an equal number of pupae of each sex were transferred to 956 mL ventilated plastic oviposition cups. Prior to adult emergence, the cups were provided with a 10% honey-water solution plus 0.1% sorbic acid via a 4 cm cotton wick in a 22 mL plastic cup. Following oviposition, freshly laid egg masses were sterilized following Singh et al. (1985) in a 5% formaldehyde solution for 20 min, soaked in water for 20 min, and air dried in a sterile area before being transferred to 96 mL plastic cups containing approximately 30 mL of artificial diet. We routinely evaluated larvae from the healthy colony to verify the effectiveness of our sterile techniques and to ensure no infections occurred.

An infected colony of *E. postvittana* was reared as above with the addition of 10^5 *N. fumiferanae postvittana* spores per mL mixed into the artificial diet. We utilized infected final instar larvae to obtain pure spore suspensions of the microsporidium by homogenizing the larvae in DI water with a plastic pestle and filtering the homogenate through nylon mesh to remove host tissues and integument. We followed the 'triangulation method' of purification by Cole (1970) to further purify the homogenate. We used a hemocytometer and phase contrast microscopy at 400x magnification to count the number of spores per mL from individual larvae taken from the infected colony. We subsequently diluted the purified spore homogenate with DI water to obtain concentrations of 10^2, 10^3, 10^4, or 10^5 spores per 2 µL for the pathology experiments.

To determine the consequences of infection for *E. postvittana*, experimental second instar host larvae were starved for 24 h in a 22 mL plastic cup prior to providing a 2mm cube of artificial diet with either 2 µL distilled water (control) or 2 µL of one of the spore concentrations from the dilution series (spore dose). After 24 h when the 2 mm cube of diet had been completely consumed, we gave all larvae a fresh 1.5 cm cube of diet (not infected), and refreshed the diet cube every week. Freshly emerged females were enclosed with two non-infected males in an oviposition cup and we collected all egg masses and stored them individually in sterile cups with

moist cotton wicks to record the number of hatched larvae and unhatched eggs per egg mass. We recorded juvenile development time and survivorship (from inoculation as second instar larvae to adult emergence), proportion of females that oviposited, and per capita viable lifetime fecundity representing the product of the number of eggs laid and the proportion that hatched. Gender was determined at pupation and could be applied to the analysis of juvenile development time, but not to juvenile survivorship. To confirm that control individuals remained uninfected, we monitored larval frass for the presence of spores at the time of pupation using smears on glass slides with 2 μL water examined under phase contrast microscopy at 400x magnification. We also followed this protocol for all infected individuals to ensure that infection occurred.

Latent period

To determine the latent period, the time interval from infection (spore ingestion) to becoming infectious (spore egestion) (Solter, 2014), we infected 20 second instar larvae with 10^3 spores as described above. After 24 h, 17 surviving larvae were transferred to individual new 22 mL plastic cups and provided fresh 2 mm cubes of diet daily. Frass was collected and monitored for the presence of spores every 24 h using smears on glass slides with 8-16 μL of water (depending of the amount of frass) and examined under phase contrast microscopy at 400x magnification.

Field prevalence and intensity of N. fumiferanae postvittana

We sampled *E. postvittana* larvae and pupae from five locations in coastal California: Santa Cruz, San Francisco, Albany, Berkeley, and Richmond for three consecutive seasons (Table 1), fall (late August-early September), late winter (late February-early March), and summer (late June-early July), to coincide with the seasonal peaks of larval activity recorded by Buergi et al. (2011). For some locations, *E. postvittana* was found on a single plant species only (Table 1) and thus 2-3 sites, 1.5 km apart, were sampled for these locations (Santa Cruz, San Francisco). For other locations, *E. postvittana* were present on 2-3 plant species and thus each plant species was sampled separately (Albany, Berkeley, Richmond). As *E. postvittana* has leaf-rolling larvae, we counted the number of leaf rolls found in a 3 min search per plant for each site or plant species from an average of 26 individual plants (ranging from 12 to 63) on each sample date. On each sampling occasion, we also collected 50 leaf rolls from a minimum of two additional plants for each site or plant species and processed them in the laboratory to determine the proportion occupied by either a larva, a pupa, or a parasitoid cocoon. We estimated population density as the proportional occupancy of the 50 leaf rolls multiplied by the average leaf rolls per min per plant. Hosts recovered from the leaf rolls collected from each site or plant species on each sample date were recorded by life-stage (larval instar or pupal stage), dipped in 5% bleach, rinsed with ddH_20, dried with a clean paper towel and stored individually in 1.5 mL sterile polypropylene tubes at -80°C for DNA extraction.

DNA was extracted from individual field-collected hosts using the modified Chelex method (Cordes et al., 2012; Huang and Solter, 2013). Whole larvae or pupae (100-500 mg size range) were rinsed with 5% Tween^{TM}20 and placed in a 2 mL microcentrifuge tube with an integrated TPE cap with 500 µL of 5% Chelex 100 Resin in 5% Tween^{TM}20 and 25 µL Proteinase K (20 mg/ml). A 5 mm stainless steel bead was added and the suspension was homogenized with a tissue lyser (Qiagen, TissueLyser II) at 20 1/s frequency for 30 s. The homogenate was briefly

centrifuged (13,000 rpm) for 30 s, and then heated for 3 h at 56 °C, followed by 1 h at 95 °C. After cooling to 4 °C, we centrifuged the samples (13,000 rpm) for 10 min and retained the supernatant for DNA purification. The Qiagen DNeasy Blood & Tissue kit was used for purification following the manufacturer's instructions but substituting EconoSpin® silica-membrane spin-columns for the Qiagen spin-columns. Samples were extracted and purified in batches of 48, including 47 field-collected hosts and one control larva from a healthy laboratory colony that was subsequently used as a non-target control (NTC) in the qPCR assays. We used a spectrophotometer (NanoDrop2000, Thermo Fisher Scientific) with AE reagent (Qiagen) as a blank to measure the concentration (ng) of the purified DNA and confirm its purity (values of ≥ 2.0 for both the 260/280 and 260/230 ratios). We diluted the purified DNA with nuclease free water to 25 ng/μL and re-measured the diluted solutions (with nuclease free water as the blank) to confirm accuracy prior to use in qPCR. Extracted, purified and diluted DNA was stored at -20 °C in 1.5 mL sterile polypropylene tubes.

We designed primers with Primer3plus (Untergasser et al., 2007) that were optimized for qPCR assays (LifeTechnologies, 2014) and specific to the *N. fumiferanae* species complex. The forward primer, NL_SSU_F 3'ATGGTTAGGAGAGAGATGAAATGTG'5 and the reverse primer, NL_SSU_R 3'TCCTCTAGCTTACGTCCTTGTT'5 (Integrated DNA Technologies), resulted in a fragment size of 105 bp. We carried out all reactions on an AB 7500 Fast Real-Time PCR system thermal cycler using the SYBR® green PCR master mix to detect the prevalence and intensity of *N. fumiferanae postvittana* (Huang and Solter, 2013). Each 20 μL reaction contained 10 μL SYBR green Master Mix, 7 μL nuclease free water, 250 nm of each primer (0.5 μL of each forward and reverse 10 μM primer), and 2 μL of 25 ng DNA sample. Conditions for qPCR were 95 °C for 20 s, followed by 40 cycles of 95 °C for 3 s and 60 °C for 30 s, followed by a dissociation stage (post-amplification melting-curve analysis) of 95 °C for 15 s, 60 °C for 60 s, 95 °C for 15 s and 60 °C for 15 s. Initial qPCR conditions and primer concentrations were optimized as above using a serial dilution series (10, 10^2, 10^3, 10^4, 10^5, 10^6, 10^7, 10^8 gene copies/ 2 μL) constructed from a plasmid 'mini-gene' (Integrated DNA Technologies, Ref ID: 124708266). We designed the plasmid to have a 2577 bp sequence covering that of the primer-specified region.

Following the optimization and verification of qPCR conditions, we redesigned the standard curve solutions to assess the number of spores in a host rather than the number of gene copies. Standard curve solutions were obtained from three independent sets of purified DNA extractions from a combination of a 5^{th} instar larva (120-130 mg in fresh weight) with 50, 10^2, 10^3, 10^4, 10^6, or 10^8 filtered and purified spores (Cole, 1970) in a 525 μL Chelex-Proteinase K solution (described above) . We used identical DNA extraction methods and the same volumes and amounts (ng) for the standard curve as for the field samples. Using this approach we determined that the number of spores from the standard curve solutions represented 1/10 the number gene copies from the equivalent plasmid dilutions.

We ran a blank, a NTC, standard curve solutions and field samples in triplicate, in fast optical 96-well reaction plates (4346906, MicroAmp™, Thermo Fisher Scientific) with optical adhesive film (4311971, MicroAmp™, Thermo Fisher Scientific). Blanks and NTCs were used to detect potential PCR contamination and unintended amplification products (Bustin et al., 2009). The blank was composed of the master mix with 2 μL nuclease free water in place of the sample. We

stored multiple small aliquots of each of the three sets of standard curve solutions to avoid degradation from numerous freeze-thaw cycles.

We analyzed all 444 field samples using the software SDS 2.2 (Applied Biosystems, Thermo Fisher Scientific), with ROX™ dye as the passive reference (LifeTechnologies, 2014) and the standard curves as a quantitative reference to quantify the prevalence and intensity of *N. fumiferanae postvittana* infection. For analysis, we adjusted the baseline (3-11) to accommodate the minimum quantification cycle (Cq) of the highest standard curve solution (10^8) (LifeTechnologies, 2014), and used the automated 0.05 as the threshold for all plates. To estimate intensity we used the mean spore concentration from those triplicates for each sample that was considered positive for spore presence. We inspected the melting curves for each well of the reaction plates and considered samples positive for *N. fumiferanae postvittana* only when the mean spore concentration was ≥ 50 spores, the melting curve had a clean peak and a melting temperature (TM) of approximately 71.63°C, and the Cq was ≤ 33. We eliminated a sample (reaction well) from a triplicate set of samples if it deviated substantially in spore concentration, melting curve, melting temperature, or Cq relative to the other two reactions. We estimated the assay efficiency for each qPCR run from $100*((10^{(-1/x)}) - 1)$ where x is the slope of the relationship between Cq and the log spore concentration of the standard curve solutions. An efficiency of 100% indicates the amount of product doubles with each cycle (Bustin et al., 2009), with a desirable range of 90-110% (LifeTechnologies, 2014).

Statistical analysis

All statistical analyses were carried out using R (R Development Core Team, version 3.1.2, 2014). We used generalized linear models (GLMs) to analyze the consequences of spore dose on the life history performance of *E. postvittana*. Spore dose (0, 10^2, 10^3, 10^4, 10^5) and gender (male or female) were used as fixed factors for the analysis of juvenile development time, while spore dose alone was a fixed factor for the analysis of juvenile survivorship and adult female performance (incidence of oviposition and viable lifetime fecundity). We plotted and analyzed the LD_{50} with a binomial model and a two-parameter log-logistic function using the *drc* package in R (Ritz and Streibig, 2005). Full models included all explanatory variables and two-way interactions, and *P* values were obtained by stepwise model simplification using likelihood-ratio tests (χ^2 – tests) in the absence of overdispersion, or *F* tests when overdispersion occurred (Crawley, 2013). We ensured that error distributions and homogeneity were appropriate by inspecting plots of residuals or standardized deviance versus predicted values, and normal quantile plots (Crawley, 2013).

Stage-structured matrix models for *E. postvittana* based on five life stages (egg, larva, pupa, pre-reproductive adult, and reproductive adult) were used to estimate the intrinsic rate of increase (*r*) for individuals exposed to either 0, 10^2, 10^3, 10^4, or 10^5 spores of *N. fumiferanae postvittana* (Caswell, 2001). The mean life history responses of females for each spore dose were used to parameterize the matrix elements of each model. The matrices of transition elements were analyzed with the *popbio* package in R to obtain estimates of *r* (Stubben and Milligan, 2007). We plotted and analyzed the rate of increase data in relation to spore dose with a four-parameter Weibull (type 1) model using the *drc* package in R (Ritz and Streibig, 2005).

For the field sampling we analyzed the population density of *E. postvittana* (counts of juveniles per min per plant) using GLMs with a Poisson error distribution and location and season as fixed factors, and a generalized linear mixed model (GLMM) with plant species as a fixed effect and season as a random effect to analyze population densities from locations that had more than one plant species (Albany, Berkeley and Richmond). We did not analyze pathogen intensity among locations due to low prevalence, but used GLMs to analyze the prevalence of *N. fumiferanae postvittana* in field populations of *E. postvittana*, with a binomial error distribution. Explanatory variables for prevalence included host density (from the same or previous season) and location. Using separate models we also analyzed the effect of season for each location and of plant species for locations that had more than one plant species (Albany, Berkeley and Richmond).

3.4 RESULTS

Life history consequences of spore dose

Juvenile development time of *E. postvittana* increased with spore dose and was significantly longer for females compared to males (Fig. 1A, Table 2). Juvenile survivorship decreased significantly with increasing spore dose (Table 2) and the median lethal dose of spores (LD_{50}) was $1.8 \pm 0.8 \times 10^4$ SE (Fig. 1B). Both the incidence of oviposition by *E. postvittana* females (Table 2) and their viable lifetime fecundity (eggs that hatched) decreased significantly with increasing spore dose (Fig. 1C, Table 2). The intrinsic rate of increase of *E. postvittana* showed an accelerating decline with increasing spore dose ($\chi^2 = 15.15$, $df = 2$, $P < 0.001$, Fig. 1D).

Latent period

The mean latent period for individuals infected with 10^3 spores of *N. fumiferanae postvittana* as second instar larvae was 12.67 days (± 2.45 SD, $n = 17$).

Field prevalence and intensity

Population densities of *E. postvittana* were influenced by a significant interaction between location and season (GLM Poisson, $n = 453$, $\chi^2 = 583.84$ $df = 12$, $P < 0.001$), but were generally higher in Berkeley and Richmond than at the other locations sampled (Fig. 2). The pattern of change between seasons was similar for Albany, Richmond and San Francisco with peak densities in the fall followed by a decline during winter, which contrasted with a general decline in population density over the sampling period at both Berkeley and Santa Cruz. We found a significant effect of plant species on population density in Albany and Richmond, but not in Berkeley (GLMM Poisson, Albany: $n = 54$, $\chi^2 = 24.00$, $df = 2$, $P < 0.001$; Richmond: $n = 99$, $\chi^2 = 90.48$, $df = 1$, $P < 0.001$; Berkeley: $n = 60$, $\chi^2 = 0.52$, $df = 1$, $P = 0.46$). In Albany, *Acacia melanoxylon* and *Genista monspessulana* had higher densities than *Baccharis pilularis* (lsmeans, $P < 0.001$), and in Richmond, *Myrica californica* had higher densities than *Ceanothus thyrsiflorus* (lsmeans, $P < 0.0001$).

The qPCR assay was sufficiently sensitive to detect 50 spores of the microsporidium (*N. fumiferanae postvittana*) in *E. postvittana* larvae, with a mean Cq of 33.15 ± 1.05 SD ($n = 21$ plates). Standard curves for the spore dilutions had a mean slope of –3.08 ± 0.08 SD, a mean

coefficient of determination (R^2) of 0.99 (± 0.01 SD), and a mean efficiency of 112 ± 4% SD (n = 21 plates). Out of the 21 reaction plates, 41% of the non-target controls and 71% of the blanks (water) had undetermined Cq values (no amplification) and/or TM values outside the acceptable range. For the non-target controls and blanks with TM values from 70 to 73 (indicating possible contamination), the mean Cq was 35.89 ± 2.20 SD and 34.59 ± 1.98 SD respectively (n = 21 plates).

Overall the prevalence of the microsporidium in field populations of *E. postvittana*, as detected by qPCR, was 5% for the 444 individuals sampled from all locations, plant species and seasons. Prevalence ranged from 0 to 15% depending on location and season (Fig. 2), and from 0 to 22% when analyzed by plant species. The intensity of infection (spore load per infected individual) ranged from 50 to 1.2 x 10^8 spores due to a single heavily infected outlier from Richmond. Excluding this individual there was a mean intensity of infection of 226.18 ± 249.61 SD spores. Peak microsporidian prevalence (22%) and peak intensity (1.2 x 10^8) occurred on *C. thyrsiflorus* in Richmond during the winter season ($n_{infected}$ = 2).

There was no interaction or significant effect of location or current host density on the prevalence of the microsporidium in *E. postvittana* populations (GLM binomial, n = 34, location x density χ^2 = 4.65, df = 4, P = 0.32, location χ^2 = 4.32, df= 4, P = 0.36, density χ^2 = 0.46, df = 1, P = 0.50, Fig. 2). However, there was a significant interaction between location and host density in the previous season for the prevalence of the microsporidium in *E. postvittana* populations (GLM binomial, n = 34, χ^2 = 14.30, df = 4, P = 0.01). Season did not have a significant effect on prevalence at the Berkeley, Richmond, Santa Cruz, or San Francisco locations (GLM binomial, Berkeley n = 6, χ^2 = 4.50, df = 2, P = 0.11; Richmond n = 6, χ^2 = 0.62, df = 2, P = 0.73; Santa Cruz n = 9, χ^2 = 1.78, df = 2, P = 0.41; San Francisco n = 9, χ^2 = 3.55, df = 2, P = 0.17). However, seasonality did have a significant effect on prevalence at the Albany location (GLM binomial, n = 7, χ^2= 7.61 df = 2, P = 0.02, Fig. 2). Host plant species did not have a significant effect on prevalence of *N. fumiferanae* in *E. postvittana* in Albany or Berkeley (GLM binomial, n = 7, χ^2 = 0.80, df = 2, P = 0.67; n = 6, χ^2 = 0.38, df =1, P = 0.54), but prevalence was significantly higher for *E. postvittana* larvae in Richmond on *C. thyrsiflorus* than on *M. californica* (GLM, binomial n = 6, χ^2 = 4.24, df =1, P = 0.04).

3.5 DISCUSSION

From the spore dose experiments under laboratory conditions there were notable fitness consequences for infection of *E. postvittana* larvae by *N. fumiferanae postvittana*. Comparing healthy larvae to larvae infected with up to 10^5 spores, we found a reduction in juvenile survivorship (from 100% to 26%), a prolongation of juvenile development time (of 9 days for females and 10 days for males), a reduction in viable lifetime fecundity (from 788 to 1) and a reduction in the intrinsic rate of increase (from 0.18 to 0.008). We estimated the LD_{50} to be 1.8 x 10^4 spores, comparable to that for *Nosema fumiferanae* in fourth instar host larvae of the spruce budworm, *C. fumiferana* (Bauer and Nordin, 1988). At low doses of spores ingested, the effects of *N. fumiferanae postvittana* were more chronic than acute, and had a greater impact on the viable lifetime fecundity of females than on other life history parameters. From our stage-structured matrix models, we were able to determine that these sublethal effects could still have

an important effect on the intrinsic rate of increase of *E. postvittana,* particularly for infections that were initiated with 10^3 spores or more.

The latent period for *E. postvittana* infected with *N. fumiferanae postvittana* was 12.7 days when second instar larvae were infected with 10^3 spores. This is shorter than the latent periods found for *Endoreticulatus schubergi* (Zwölfer), *N. lymantriae* Weiser*, and Vairimorpha disparis* (Timofejeva) infection in *L. dispar* L. larvae (13.5, 15.1, and 15.0 days respectively, Goertz and Hoch, 2008), and for *N. fumiferanae* infection in *C. fumiferana* larvae (15.0 days, Campbell et al. 2007). However, host larvae in these other studies were infected as third instars rather than second instars which may have affected the spore replication times. The shorter latent period, combined with the dose-dependent sublethal and lethal effects of *N. fumiferanae postvittana* suggest that it could have a similar effect on field populations of *E. postvittana* as the related isolate, *N. fumiferanae,* which can achieve over 80% infection in populations of *C. fumiferana* (Thomson, 1960a).

To determine how the potential impact of *N. fumiferanae postvittana* on *E. postvittana* larvae in the laboratory translates to field conditions, we monitored five locations to estimate the population densities of the host, and the prevalence and intensity of the pathogen. The field sampling demonstrated that the population densities of *E. postvittana* varied with location, season and plant species, and that population densities in San Francisco and Santa Cruz in 2013/14 had further declined from those documented in the same two locations from 2009 to 2012 (Bürgi et al., 2015). We detected the microsporidium in hosts from all locations, seasons and plant species. Prevalence was low (5% overall) and accompanied by a low intensity of infection (mean of 226 spores). As the qPCR efficiency averaged a little over 110%, indicating potential inhibition and lowered detection sensitivity (LifeTechnologies, 2014), low spore loads (< 50 spores) are likely to have been missed. However, the detection level from our qPCR analyses was still much greater than that possible by dissection and light microscopy (Refardt and Ebert, 2006), and the results from our spore dose experiment indicate that infection loads of less than 50 spores are unlikely to impact the fitness of infected host individuals.

Prevalence did not appear to be influenced consistently by host density or by season. Although there was no overall seasonal effect on prevalence, there was evidence of a seasonal lag in the effect of host density on the prevalence of the pathogen at the Albany location, which is consistent with similar observations on host-pathogen interactions for other Microsporidia (Kohler and Hoiland, 2001; Lewis et al., 2009). There was also evidence of a host plant effect, particularly at the Richmond location, where the prevalence of *N. fumiferanae postvittana* was higher for host larvae on *C. thyrsiflorus* than on *M. californica*, perhaps due to the chemical and/or physical characteristics of these host plants (Cory and Hoover, 2006). Other location-specific characteristics that can influence host-microsporidian interactions, such as irrigation and precipitation (Milks et al., 2008), and interactions with other natural enemies (Eveleigh et al., 2012), were not investigated in this study.

As the estimated LD_{50} of 1.8×10^4 spores found in the laboratory was considerably higher than the mean infection of 226 spores observed for field-infected individuals, larvae of *E. postvittana* under field conditions in California would be more likely to experience sublethal than lethal effects from this pathogen. The low prevalence and intensity of *N. fumiferanae postvittana* at our

field locations may have been due to the relatively low population densities and patchy spatial distributions of *E. postvittana* larvae, and to the relatively low mobility of the larval stage due to its leaf-rolling habit. Horizontal transmission of *Nosema* spores primarily occurs through contact with infected individuals and/or infected frass (Solter et al., 2012a; Solter, 2014). Thus the risk of infection for *E. postvittana* via horizontal transmission is likely low in the field unless larvae share leaf rolls, an event that is more common at higher larval densities (Bürgi et al., 2015).

The low prevalence and intensity of *N. fumiferanae postvittana* infection found in populations of *E. postvittana* in California provides little evidence that this microsporidium currently contributes to the suppression of *E. postvittana* populations in the field. However, a low prevalence and intensity is typical of microsporidian infections in other insect host populations. Infections with the potential to suppress their host populations typically have higher prevalence and intensity than observed in the present study system, although Microsporidia are only rarely epizootic (Solter et al., 2012a). For example, the prevalence of *N. fumiferanae* can reach over 80% in populations of *C. fumiferana* (Thomson, 1960a), and infections of *Ostrinia nubilalis* (Hübner) by *N. pyrausta* (Paillot), can reach over 60% (Hill and Gary, 1979; Lewis et al., 2009).

Although our laboratory study demonstrated strong negative effects of the microsporidium, *N. fumiferanae postvittana* on the life-history performance of *E. postvittana*, combined with a relatively short latent period, indicating the potential for host population suppression, we did not find any evidence for strong impacts in the field. The low levels of infection by *N. fumiferanae postvittana* in the field contrasts with the greater than expected levels of parasitism by resident parasitoid wasps on this exotic host species (Bürgi et al., 2015). As discussed by Levine et al. (2004), although biotic resistance by resident natural enemies is unlikely to repel invasions completely, these biotic interactions, including that of *Nosema fumiferanae postvittana*, can still be of importance in reducing the abundance and spread of exotic species, such as *E. postvittana*.

3.6 TABLES

Table 1. Locations and dates sampled for *Epiphyas postvittana* and associated microsporidian *Nosema fumiferanae postvittana* in California, USA with number of sites per location and plant species sampled at each location. All plants were sampled on all dates.

Location	Sites	Host plants	Dates
San Francisco	2	*Leptospermum laevigatum*	2013: Jul 10, Sep 12 2014: Mar 10, Jul 16
Santa Cruz	3	*Arctostaphylos densiflora*	2013: Jul 16, Aug 29 2014: Feb 24, Jun 30
Richmond	1	*Myrica californica, Ceanothus thyrsiflorus*	2013: Jul 2, Sep 2 2014: Mar 12, Jul 14
Albany	1	*Acacia melanoxylon, Baccharis pilularis, Genista monspessulana*	2013: Jul 18, Sep 4 2014: Mar 3, Jul 9
Berkeley	1	*Euryops pectinatus, Pittosporum tobira*	2013: Jul 23, Sep 9 2014: Mar 5, Jul 7

Table 2. Statistical analysis of the effect of the microsporidium, *Nosema fumiferanae postvittana,* on the life history performance of *Epiphyas postvittana*. *N* represents the number of replicates for each of the spore loads (0, 10^2, 10^3, 10^4, 10^5 spores) ingested by second instar *E. postvittana* larvae. The juvenile stage represents the period from larval infection to adult emergence, and viable lifetime fecundity represents the number of hatched eggs produced in the lifetime of an individual female.

Model	*N* (0-10^5)	GLM family, statistic	*df*	*P*
Juvenile survivorship	69, 48, 47, 74, 47	binomial, $\chi 2$		
Spore load		95.57	4	< 0.001
Juvenile development time	66, 41, 34, 48, 12	Poisson, $\chi 2$		
Spore load		60.71	4	< 0.001
Gender		15.50	1	< 0.001
Oviposition incidence	35, 21, 16, 27, 9	binomial, $\chi 2$		
Spore load		20.02	4	< 0.001
Viable lifetime fecundity	33, 20, 11, 22, 2	Poisson, $\chi 2$		
Spore load		20422.00	4	< 0.001

3.7 FIGURES

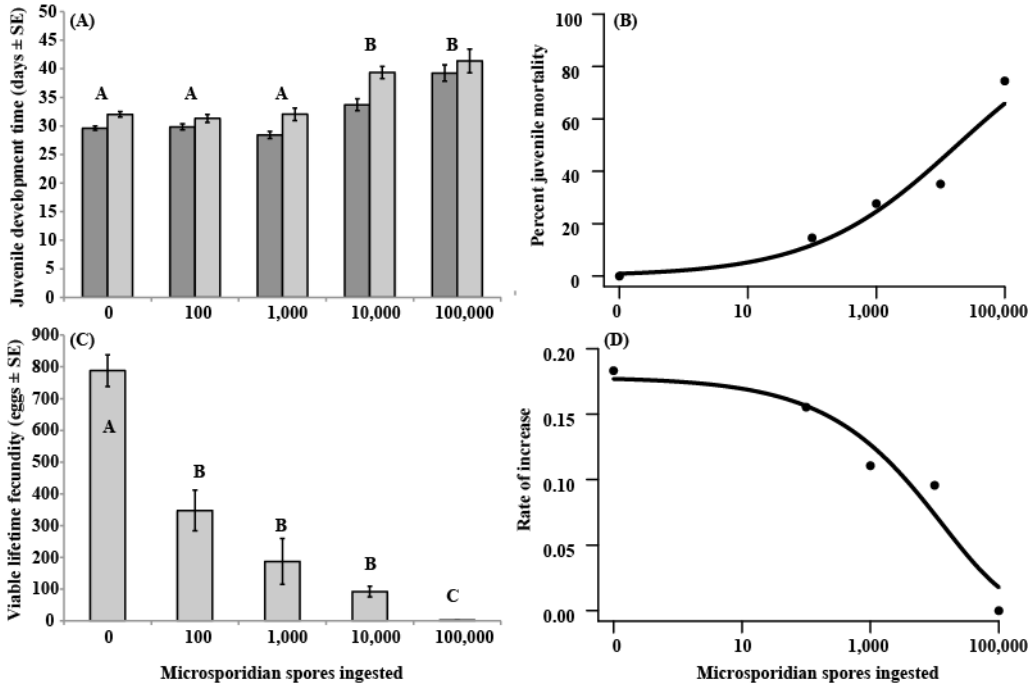

Figure 1. Mean (± SE) effects of the number of spores of *Nosema fumiferanae postvittana* ingested by 2nd instar larvae of *Epiphyas postvittana* on (A) juvenile development time for males (dark grey) and females (light grey), (B) juvenile mortality, y = 100/(1+exp(-0.39 *(ln(x) – ln(18178)))), (C) viable lifetime fecundity and (D) intrinsic rate of population increase, y = 0.18*exp(-exp(0.37*(ln(x)-ln(14065)))). The x-axis scales in (B) and (D) are log-transformed.

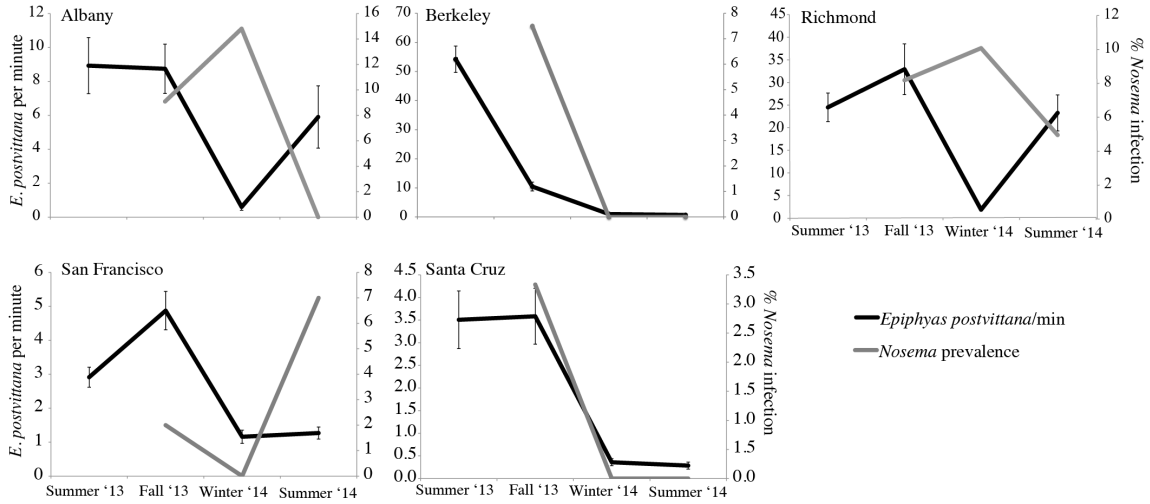

Figure 2. Density (mean number of occupied leaf rolls per min per plant) of juvenile *Epiphyas postvittana*, and prevalence of *Nosema fumiferanae postvittana*, at five locations in coastal California for 2013-14. Note the difference in axis scales between locations.

CHAPTER 4

MULTITROPHIC INTERACTIONS AND HOST PLANT EFFECTS ON THE ENEMY-FREE SPACE OF AN EXOTIC, GENERALIST HERBIVORE

4. 1 ABSTRACT

In this study we examine multitrophic interactions among an exotic generalist herbivore in California, *Epiphyas postvittana* (Lepidoptera: Tortricidae), exotic and native plants, and resident enemies that include larval parasitoids and a microsporidian pathogen. Based on a common garden experiment conducted in the field, we found that parasitism of egg masses varied by a factor of 4.7 between host plant species. Plant architecture influenced the extent of egg parasitism with a higher risk of parasitism on taller plants than shorter plants. Larval parasitism also varied among plant species by a factor of 2.1 with a higher risk of parasitism for larvae on shorter plants than taller plants. In the laboratory, we conducted experiments to further examine which aspects of plant architecture and leaf morphology may have influenced parasitism rates for egg masses in the field. Time taken for a parasitoid to find an egg mass varied among plant species, but did not correspond to rates of egg mass parasitism in the field. In addition, the probability of contacting an egg mass on leaves of *Plantago lanceolata* decreased with trichome density. In the laboratory, we also found significant effects of diet quality on the fitness of both healthy and microsporidian-infected *E. postvittana*, with evidence of synergistic effects between diet and infection. Overall, there was a significant effect of host plant on the extent of enemy-free space for *E. postvittana* and this is discussed in the context of plant origin and domestication, and novelty in multitrophic interactions.

4. 2 INTRODUCTION

Global transportation and trade have combined synergistically with the onset of global warming to facilitate the spread and colonization of many invasive species (Crowl et al., 2008). Although the majority of invasive species frequently escape their natural enemies (Keane and Crawley, 2002; Mitchell and Power, 2003; Torchin et al., 2003), occasionally, invasive species will encounter biotic resistance via novel resident enemies (Elton, 1958; Maron and Vila, 2001; Kimbro et al., 2013). As top-down effects from enemies do not occur in isolation, it is of increasing interest to understand the outcomes of multitrophic interactions among resident species and novel invasive species (Harvey et al., 2010; Chabaane et al., 2015). Novel multitrophic interactions, involving one or more exotic species, may have very different outcomes than those involving co-adapted resident species (Cox, 2004; Tanaka et al., 2007; Fortuna et al., 2013; Chabaane et al., 2015).

Multitrophic interactions among native plants, insect herbivores and their enemies (predators, parasitoids and pathogens) have been studied extensively, primarily from a perspective of the bottom-up effects of plant quality and the top-down effects of enemies on the performance and diet breadth of herbivores (Price et al., 1980; Schoonhoven et al., 2005; Singer and Stireman, 2005; Mooney et al., 2012). Such studies have generated three key hypotheses regarding herbivore performance; that host plant specialists exhibit greater physiological efficiency than generalists, that specialists experience more enemy-free space than generalists on shared host plants, and that herbivores experience greater exposure to enemies on lower versus higher quality plants (Mooney et al., 2012). Multitrophic interactions can be influenced by abiotic factors and a variety of plant traits including architecture, morphological adaptations, nutritional quality, and the direct and indirect effects of chemical defenses (Gols, 2014; Reinecke and Hilker, 2014).

Both plant architecture (Andow and Prokrym, 1990) and plant volatiles (Dicke and Baldwin, 2010; Pierik et al., 2014) can influence the foraging success of herbivores and their natural enemies (Gols, 2014). In addition, secondary plant metabolites and plant nutritional quality can also affect the growth and survivorship of herbivores, and consequently their suitability for the growth, survival and reproduction of their natural enemies (Gols, 2014). On the one hand, secondary plant metabolites can have a positive influence on natural enemies by stressing insect herbivores and either increasing the risk of enemy attack (Benrey and Denno, 1997), or decreasing resistance to enemy attack (Cory and Hoover, 2006; Bukovinszky et al., 2009; Gassmann et al., 2010). On the other hand, these metabolites can also have a negative influence on natural enemies through increased resistance of herbivores to enemy attack (Singer et al., 2009; Gassmann et al., 2010), or reduced transmission rates in the case of pathogens (Richardson et al., 2015). Thus the direct and indirect effects of plant traits are not independent and may interact in influencing multitrophic interactions (Gols, 2014). For example, there is often a trade-off between the nutritional quality of a plant for an herbivore and the extent of enemy-free space that it provides (Price et al., 1980; Singer et al., 2004; Gassmann et al., 2010).

More recently, it has become clear that there is a need to study multitrophic interactions in more realistic field settings, that extend beyond simple tritrophic systems based on domesticated crops to include natural plants (Gols, 2014; Harvey et al., 2015) and novel interactions with exotic species (Harvey et al., 2010). Here, we examine the consequences of multitrophic interactions

among an exotic generalist herbivore, a variety of herbaceous and woody host plants, and several resident enemies including parasitoid wasps and a pathogen. The light brown apple moth, *Epiphyas postvittana* (Walker) (Lepidoptera: Tortricidae), is a leaf-roller native to Australia with a host range of over 500 plant species, and was confirmed to be present in California in 2006 (Brown, 2007; Suckling et al., 2014). Since its introduction to California, *E. postvittana* has been colonized by a wide range of resident parasitoids, including the egg parasitoid, *Trichogramma fasciatum* (Perkins) and the larval parasitoids *Meteorus ictericus* Nees and *Enytus eureka* (Ashmead) (Wang et al., 2012; Bürgi and Mills, 2014; Bürgi et al., 2015). It has also been impacted by generalist predators (Hogg et al., 2013) and a newly discovered microsporidian pathogen, *Nosema fumiferanae postvittana* subsp. n. (hereafter: *N. fumiferanae postvittana*) (Chapter 2). As *E. postvittana* is a generalist herbivore, feeding on plants from a wide range of families, particular host plants may have an important influence on its performance and potential for enemy-free space with respect to resident parasitoids and pathogens.

To determine the influence of host plants on parasitism of *E. postvittana* eggs and larvae by resident parasitoids we used a common garden experiment with both native and exotic plant species in a heterogeneous urban landscape. In addition, we monitored parasitism of host egg masses by *T. fasciatum* on excised leaves in the laboratory to examine the consequences of leaf characteristics for parasitoid foraging success. For these two experiments we hypothesized that egg parasitism would be influenced by morphological traits of the host plants, as has been shown previously for other *Trichogramma* species that forage by walking on the plant surface (Andow and Prokrym, 1990; Romeis et al., 2005; Olson and Andow, 2006). In contrast, we hypothesized that larval parasitism would be less likely to be influenced by plant architecture, since plant volatiles play a greater role in host location for parasitoids that forage in flight (Dicke and Baldwin, 2010). In addition, we predicted that both egg and larval parasitism in the field would be affected by climatic factors such as wind speed and temperature (Reinecke and Hilker, 2014).

To determine the effect of host plant quality on the performance of *E. postvittana*, we monitored their growth, survival and reproduction when fed on a variety of diets under laboratory conditions. To examine whether diets differ in their potential to provide protection from natural enemies, we compared the effect of diet quality on healthy *E. postvittana* larvae and those infected with the microsporidian, *N. fumiferanae postvittana*. We hypothesized that there would be a positive fitness gain for healthy *E. postvittana* larvae when feeding on higher quality diets and that stress from lower quality diets would further reduce the fitness of infected *E. postvittana* (Gassmann et al., 2010).

4. 3 MATERIALS AND METHODS

Host plants

We studied nine host plants commonly found in the San Francisco Bay Area of California and known to be host plants used by *E. postvittana* (Brockerhoff et al., 2011). The host plants were comprised of four crops, apple (*Malus domestica* Borkh.), grape (*Vitis aestivalis* Michx.), raspberry (*Rubus idaeus* L.) and strawberry (*Fragaria vesca* L.), two herbaceous plants, plantain (*Plantago lanceolata* L.) and beardtongue penstemon (*Penstemon campanulatus* (Cav.) Willd.), and three woody shrubs, Australian tea tree (*Leptospermum laevigatum* (Gaertn.) F. Muell.), lilac

vine (*Hardenbergia violacea* Schneev.), and manzanita (*Arctostaphylos densiflora* (Howard McMinn) M.S. Baker). We grew plantain and beardstongue penstemon from seed, apple, grape, raspberry, and strawberry from purchased and grown from root cuttings, and Australian tea tree, lilac vine and manzanita as potted plants purchased from local nurseries in 2010. We controlled for any potential belowground effects by using potted plants with equivalent soil and nutrient conditions. All plants were grown in 3.8 L plastic pots (18.8 L for apple) in a standard potting soil (Super Soil®, Scotts Miracle-Gro, Marysville, Ohio), and maintained in a greenhouse at 22 ± 1.5°C and a 16D: 8L h photoperiod using extended daylight from metal halide lamps. Plants were watered daily and provided with Peters Professional Water Soluble 20:20:20 Fertilizer (Scotts-Sierra Horticultural Products, Marysville, Ohio) twice weekly. Plants were sprayed weekly with Ecotrol EC (EcoSmart Technologies, Inc. Franklin TN 37067) with Triton X-100 surfactant (Sigma-Aldrich, St. Louis MO 63103) at the label rates to prevent spider mite damage. Plants intended for use in experiments were not sprayed the week prior to experimentation. As Ecotrol is a contact- pesticide we did not expect residual impacts on the study organisms following this minimum post-exposure period.

Host, parasitoid and pathogen colonies

All colonies were maintained at 20°C, a 16:8 h L:D photoperiod, and a relative humidity of 60%, and all laboratory experiments were conducted under the same conditions. A laboratory colony of *E. postvittana* was established from larvae collected from Santa Cruz, California in 2007. Larvae were reared on an artificial bean-based diet developed by Cunningham (2007). Upon pupation, an equal number of pupae of each sex were transferred to 956 mL ventilated plastic oviposition cups (Fabri-Kal, Kalamazoo MI). Prior to adult emergence, the cups were provided with 10% honey-water with 0.1% sorbic acid via a 4 cm cotton wick in a 22 mL plastic cup (SOLO, Highland Park, IL). Following oviposition, freshly laid egg masses were sterilized following Singh et al. (1985) in a 5% formaldehyde solution for 20 min, soaked in water for 20 min, and air dried in a sterile area before being transferred to 96 mL plastic cups (SOLO) containing approximately 30 mL artificial diet.

A colony of *T. fasciatum* was established from parasitized egg masses found in Albany and Berkeley, California from 2011-2014. Adult parasitoids were reared on non-sterilized fresh egg masses of *E. postvittana* in ventilated glass vials with 50:50 honey-water streaked on the sides of the vial. We used naïve female parasitoids that were less than 24 h old for all laboratory experiments.

A colony of the microsporidian, *N. fumiferanae postvittana*, was maintained on *E. postvittana* larvae that were reared as above for the healthy colony, with the addition of 10^5 *N. fumiferanae postvittana* spores per mL of purified homogenate mixed into the artificial diet (Chapter 2). We utilized infected final instar larvae to obtain pure spore suspensions of the microsporidium by homogenizing the larvae in DI water with a plastic pestle and filtering the homogenate through nylon mesh to remove host tissues and integument. We followed the 'triangulation method' of purification by Cole (1970) to further purify the homogenate. Separate insectaries were used for the healthy and infected colonies and for the experiments. We used 10% bleach to sterilize all tools, containers and surfaces prior to use.

The influence of host plant on parasitism by resident parasitoids in the field

We conducted the field study at San Francisco State University, Daly City, California. The field site consisted of six blocks, each block containing one potted plant of each of the nine species arranged in a randomized pattern. We removed all flowers prior to the start of the experiment to avoid any differential attraction of parasitoids to floral resources. Plants were watered every other day at 05:00 h for 10 min, with a drip rate of 3.8 L per h. Plants were spaced 0.5 m apart within blocks and the blocks were separated by 1 m. We exposed either 10 egg masses (~300 eggs) of *E. postvittana* per plant or 70 ± 10 first-instar larvae per plant to parasitism in the field on each of the plant species in each experimental block. We exposed egg masses on four occasions (June and August 2010, June and July 2011), and first-instar larvae on six occasions (July-October 2010, and June-August 2011). Both life stages were exposed in the field for approximately 1.5 weeks, depending on variation in degree-day accumulation. Exposure intervals for egg masses and larvae were set at 110.10 and 165.7 (half the complete larval development period) degree days above a threshold of 7.5 °C (from Danthanarayana, 1975). Degree-days were monitored from a local weather station (Earth Networks-WeatherBug database, San Francisco State University), which was also used to record mean daily temperature and wind speed. Following field exposure, we measured plant height and width, and counted the number of branches and leaves for each individual plant. In addition, we measured average leaf surface area and average trichome density for each plant species. We monitored parasitoid emergence daily from individual *E. postvittana* egg masses in glass vials with ventilated plastic lids, and from individual larval hosts in 96 mL plastic cups (SOLO) with 30 mL artificial diet. We recorded the number of parasitized and non-parasitized hosts from each potted plant. For the egg mass exposures, a total of 7 of the 216 plants were excluded from further analysis due to damaged or missing egg masses from having fallen over during the exposure period. For the larval exposures, just over half (165) of the potted plants were excluded from analysis as they had less than 10 host larvae present at the end of the exposure period. Yellow jacket wasps were noted to be responsible for the loss of some larvae during the exposure period. The number of replicates used for statistical analyses are presented in Tables 1 and 2.

Influence of host plant on the foraging success of T. fasciatum under laboratory conditions

The influence of different host plants on the foraging success of *T. fasciatum,* was determined in the laboratory using excised leaves or stems of the same nine plant species used for the common garden experiment in the field. Prior to conducting the laboratory experiments, we enclosed six female and two male *E. postvittana* in mesh nylon bags on potted plants for 48 h to permit oviposition. Following oviposition, we excised either individual leaves or short stems (for manzanita and Australian tea tree that have much smaller leaves) that had an egg mass of a suitable size (25 eggs ± 5) oviposited more than 5 cm from the apex. The size of the excised leaves or stems was selected to maintain an approximately similar leaf surface area among plant species. We removed any extra egg masses using a damp paper towel, and wiped dry. Leaf petioles or stems were inserted into 5 mL glass vials filled with water, and secured with cotton wool before being placed in a 956 mL ventilated plastic container (Fabri-Kal). Using a damp, fine-tipped paintbrush, we placed a naïve female *T. fasciatum* at the base of the leaf or stem and observed the female for 3 h. We recorded the proportion of females that made contact with an egg mass for enough time to parasitize at least one host egg (success of contact, N =118), and the

time it took a female to find the host egg mass (search time, N = 66). Replicates were lower for analysis of search time as only 56% of all females made contact with an egg mass within the 3 h period. Following the experiment, females were saved in 70% ethanol and their hind tibia lengths were measured as estimates of adult size (Godfray, 1994). We also recorded the distance to the egg mass from the initial placement of each female, the number of leaves in each container, and the total leaf surface area.

To more specifically determine the effect of trichome density, controlling for plant species, on the foraging success of *T. fasciatum* we repeated the above experiment with an additional 22 plantain leaves (*P. lanceolata*) which vary extensively in trichome density. We recorded the same measurements as in the previous experiment, but used only one leaf per container and additionally counted the number of trichomes on both the dorsal and ventral sides of each leaf in 1 cm transverse bands across the lower, middle and upper sections of the leaf.

The influence of diet and infection status on the life history performance of E. postvittana under laboratory conditions

We used a hemocytometer (#3120, Hausser Scientific, Horsham, PA) and phase contrast microscopy at 400x magnification to count the number of spores per mL from individual larvae taken from the infected colony. We subsequently diluted the purified spore homogenate with DI water to obtain a concentration of 5 x 10^2 spores per µL. Experimental second-instar larvae of *E. postvittana* were starved for 24 h and then provided with a 2 mm cube of artificial diet coated with either 2 µL distilled water (control) or 2 µL *N. fumiferanae postvittana* spore solution (infected treatment). After 24 h when the 2 mm cube of diet had been completely consumed, the experimental larvae were assigned to a diet treatment for further rearing.

To compare the effects of diet quality on the performance of healthy *E. postvittana* larvae and those infected by *N. fumiferanae postvittana*, groups of five larvae were fed a diet that consisted either of excised leaves of one of five plant species (apple, Australian tea tree, manzanita, plantain or strawberry) or artificial diet (containing no secondary plant metabolites) that was provided either continuously (full) or interrupted by starvation for two out of every four days (partial). The groups of healthy and infected larvae were reared in 500 mL ventilated plastic containers (Newspring®) and kept in separate insectaries to avoid cross infection. Excised leaves were used for each of the plant species, with their stems inserted into small 7 mL glass vials (1.2 x 4.5 cm) of sterile water and plugged with cotton wool. For the two artificial diet treatments, we provided a 7 cm diameter by 1 cm depth circle of artificial diet in the middle of the plastic container. We refreshed all diets every four days or earlier if needed (with the exception of the partial artificial diet) to ensure that experimental larvae were provided with sufficient fresh food. To confirm that healthy individuals remained uninfected, we monitored larval frass for the presence of spores for both healthy and infected individuals at the time of pupation (or earlier if larval mortality occurred) by examining smears in 2 µL water on glass slides under phase contrast microscopy, 400x magnification. Survivorship and development were monitored daily and upon pupation the pupae from each group were transferred to small, sterile 22 mL plastic cups (SOLO) until adult emergence. Single freshly-eclosed females from each group of survivors were individually placed with two uninfected males in oviposition cups to determine their lifetime production of egg masses. Egg masses were stored in sterile cups with moist cotton

wicks to record the proportion of egg hatch. For all treatments, we recorded juvenile (larval plus pupal) development time and survivorship, gender, and the per capita lifetime production of viable eggs (lifetime fecundity multiplied by proportion of eggs that hatched). Each diet treatment (full and partial artificial diet, and the five plant species) was replicated 10 times for both healthy and infected larvae, but as mortality occurred throughout the experiment the level of replication for individual measurements varied and is presented in Table 5.

Statistical analysis

Statistical analyses were carried out using R version 3.1.2 (R Core Team, 2014) and were based primarily on generalized linear models (GLMs) and generalized linear mixed models (GLMMs). Standard link functions were used for the GLMs and error distributions were selected to best represent the measurement variables analyzed (Poisson for counts, binomial for proportions, quasibinomial for overdispersed proportions and quasi-Poisson for overdispersed counts). We ensured that error distributions were appropriate by inspecting plots of residuals or standardized deviance versus predicted values, and normal quantile plots (Crawley, 2013). Full GLM and GLMM models included all explanatory variables and interactions, and stepwise model simplification was performed manually using likelihood-ratio tests, or *F*-tests for the quasi-binomial and quasi-Poisson error distributions (Crawley, 2013). Non-significant interactions are not presented in the results. Pairwise comparisons of levels within treatments were performed with Tukey's posthoc tests in the 'multcomp' package vs. 1.3-9 (Hothorn et al., 2008). Pairwise comparisons among diets for infected and healthy larvae were corrected for heteroscedasticity with the 'sandwich' package vs. 2.3-4 (Zeileis, 2004; Zeileis, 2006). All pairwise comparisons were based on 95% CI.

To examine the influence of host plants on parasitism in the field, GLMMs were used with plant species as a fixed effect, and block number nested within sample date as random explanatory variables. We subsequently used GLMs to analyze the effect of plant architecture (height, width, number of branches and leaves, average leaf surface area, and average trichome density) and climate (temperature and wind speed) on average parasitism rates for each plant species across the six replicate blocks for each sample date. To analyze the effect of host plant on the foraging success (success of contact, search time) of *T. fasciatum* in the laboratory, explanatory variables in the GLMs included plant species, number of leaves, leaf surface area, trichome density, initial distance of the female from the egg mass, and parasitoid hind tibia length.

We utilized GLMs to determine the effects of diet quality and microsporidian infection on mean survivorship, juvenile development time and lifetime fecundity of viable eggs, with the last two measurements based on median values from each rearing group of five larvae. Diet, infection status and gender were used as explanatory variables. As gender identity could only be determined at pupation, it could be used for analysis of larval development time, but not for larval survivorship.

To estimate the intrinsic rate of increase (r) of a population exposed to each combination of diet and infection treatments we used stage-structured matrix models based on five life stages of *E. postvittana*: egg, larva, pupa, pre-reproductive adult, and reproductive adult (Caswell, 2001). The life table response data collected from each diet treatment were used to parameterize the

matrix elements of each model, based on the measurements presented in the supplementary materials (Table S4.1). The matrices of transition elements were analyzed with the *popbio* package in R (Stubben and Milligan, 2007) to estimate the intrinsic rate of increase, *r*, from the natural log of the finite rate of increase (λ).

4.4 RESULTS

The influence of host plant on parasitism by resident parasitoids in the field

The mean percent of *E. postvittana* egg masses parasitized by *T. fasciatum* varied from 2% ± 1% (SE) for grape, plantain and raspberry to 18% ± 6% (SE) for penstemon in the common garden experiment (Fig. 1) with a significant effect of plant species (Table 1). Egg masses on apple, lilac vine and penstemon plants were more likely to be parasitized than egg masses on grape, manzanita, plantain, raspberry and strawberry (Fig. 1). Egg masses on penstemon were additionally more likely to be parasitized than egg masses on Australian tea tree. The probability of egg mass parasitism was also influenced by two measures of plant architecture, increasing significantly with plant height (Fig. 2) and showing significant, but inconsistent, variation with leaf surface area (Table 1).

We also found a significant effect of plant species on larval parasitism rates in the common garden experiment (Table 2), with parasitism varying from 4% ± 3% (SE) for penstemon to 35% ± 7% (SE) for plantain (Fig. 1). Larvae from grape and plantain had significantly higher parasitism rates than larvae on apple and lilac vine with other plant species being intermediate (Fig. 1). Among the 20% of larval hosts that were parasitized across all of the field trials (N = 409), 77% were parasitized by *Enytus eureka,* 21% were parasitized by *Meteorus ictericus,* 2% of the parasitoid cocoons failed to emerge for identification, and < 1% were parasitized by a tachinid sp. In contrast to parasitism of egg masses, plant height significantly decreased the probability of larval parasitism (Fig. 2) and no other measures of plant architecture were significant (Table 2). The probability of larval parasitism was influenced by a significant interaction between temperature and wind speed, with parasitism increasing with higher temperatures and decreasing with higher wind speeds.

The influence of host plant on the foraging success of T. fasciatum under laboratory conditions

The probability of *T. fasciatum* contacting a host egg mass under laboratory conditions was not influenced by plant species, plant architecture, or parasitoid size (Table 3). However, for those parasitoids that did make successful contact with the egg mass, the search time (min) required to find the egg mass varied significantly with plant species (Table 3), from 14.40 ± 9.22 min (SE) on lilac vine to 90.00 ± 24.19 min (SE) on Australian tea tree (Fig. 3A). Search times for the egg mass were significantly longer on Australian tea tree, manzanita and raspberry compared to those on grape and strawberry (Fig. 3A). The variation in search time among plant species was not attributable to the measured leaf characteristics or to parasitoid size (Table 3).

When separately tested on plantain (*Plantago lanceolata*) leaves with varying trichome densities, *T. fasciatum* showed a significant reduction in the success of contacting the host egg mass on leaves with higher trichome densities (Fig. 3B) and a marginal increase with a larger surface area

(Table 4). For parasitoids that did contact the host egg mass, search time was not influenced by leaf morphology or parasitoid size (Table 4).

The influence of diet and infection status on the life history performance of E. postvittana under laboratory conditions

All statistical analyses of the effects of infection status and diet on the life history performance of *E. postivittana* are presented in Table 5. Infected individuals had significantly lower juvenile survivorship compared to healthy individuals for all diets and there was significant variation in juvenile survivorship among diets for both healthy and infected hosts. Healthy individuals had greater survivorship on the full artificial diet compared to the other six diets and infected individuals had lower survivorship on strawberry compared to the other six diets (Fig. 4A). Juvenile development time did not differ between infected and healthy treatments, but was significantly affected by diet, and differed significantly with gender (Table 5, Fig. 4B). Juvenile development time was longer on Australian tea tree compared to the full artificial diet, apple, manzanita and plantain. Larvae reared on manzanita had a longer juvenile development time than those reared on the full artificial diet or apple (Fig. 4B).

Lifetime fecundity of viable eggs was influenced by an interaction between infection status and diet, indicating that the response to diet quality differed between healthy and infected individuals (Fig. 4C). Among the healthy individuals, those reared on the full artificial diet, apple, manzanita, and plantain had a higher lifetime production of viable eggs than those reared on the partial artificial diet and Australian tea tree. Healthy individuals reared on manzanita additionally had a higher lifetime production of viable eggs than those reared on strawberry. There were insufficient replicates to statistically compare the lifetime fecundity of viable eggs among diets for the few surviving infected individuals. However, infected individuals reared on the full artificial diet showed a trend toward higher lifetime production of viable eggs than those reared on all other diets (Fig. 4C). As an integrated index of population growth the intrinsic rate of increase (r) could not be tested for statistical effects of diet or infection status, but was lower for infected individuals compared to controls, and also varied among diets (Fig. 4D). The reduction in intrinsic rates of increase between healthy and infected larvae was notably less for the two artificial diets than for any of the plant species tested.

4.5 DISCUSSION

Based on a common garden experiment conducted in the field, we examined multitrophic interactions involving both native (or resident) and exotic species. We found that egg masses of the exotic leaf-roller *E. postvittana* on apple, lilac vine and penstemon plants were on average 4.7 times more likely to be parasitized by *T. fasciatum* than egg masses on grape, manzanita, plantain, raspberry and strawberry. This supports previous findings that parasitism of host species by *Trichogramma* parasitoids can vary greatly depending on host plant species (Romeis et al., 2005). As expected, plant architecture influenced the extent of parasitism with plant height being the most significant indicator of parasitism risk. Egg masses of *E. postvittana* on taller plants had a higher risk of parasitism than egg masses on shorter plants. Taller plants may have greater apparency to certain *Trichogramma* spp. as Thorpe (1985) also found higher parasitism rates for *T. minutum* on eggs of *Heliothis virescens* (Fabricius) placed at greater heights on plants

in the field. These results provide additional support to the idea that small parasitoids, such as *Trichogramma* spp., are more likely to rely on visual cues while searching for hosts, and are more habitat specific than host specific (Romeis et al., 2005). We also found that parasitism of *E. postvittana* larvae varied significantly among plants in the common garden experiment. Host larvae on grape and plantain had on average 2.1 times greater parasitism rates compared to larvae on apple and lilac vine. However, in contrast to the positive relationship between plant height and parasitism risk for egg masses, larvae on shorter plants had a higher risk of parasitism by the generalist larval parasitoids, *M. ictericus* and *E. eureka*, than larvae on taller plants. Although plant height had a significant effect on parasitism rates of both egg masses and larvae in the field, there remains a potential confounding effect of plant species and plant height. Future studies could compare plants of different sizes within a species to more clearly discern the effect of plant height on parasitism rates of hosts by parasitoid wasps. In addition to the effect of plant species and height on the parasitism of larvae, we also found a significant interaction between wind speed and temperature on larval parasitism rates in the field, which may have been due to the effects of climate on the distribution and evaporation rates of plant volatiles and on parasitoid foraging (Reinecke and Hilker, 2014).

In the laboratory, we conducted experiments to further examine which aspects of the architecture and leaf characteristics of the host plants may have influenced parasitism rates for egg masses in the field. The search time for *T. fasciatum* to find an egg mass varied among plant species, with females finding egg masses 6.4 times faster on lilac vine leaves than on Australian tea tree leaves. When controlling for plant species, the probability of *T. fasciatum* contacting an egg mass on plantain leaves decreased with increasing trichome density. Leaf surface structures such as trichomes can impede the movement of small parasitoids and our observations are consistent with findings from previous studies on the foraging behavior of *Trichogramma* spp. (Lukianchuk and Smith, 1997; Romeis et al., 2005; Olson and Andow, 2006).

In comparing the results from our laboratory and field studies of egg mass parasitism, plant species that resulted in longer search times for *T. fasciatum* in the laboratory did not necessarily correspond to those that had lower rates of egg mass parasitism in the field. For example, as search times for egg masses in the laboratory were shortest for grape, lilac vine and strawberry, one would predict that parasitism rates of egg masses in the field would be the greatest on these host plants. Our observations indicate that while this was the case for lilac vine, parasitism rates for grape and strawberry were among the lowest observed in the common garden experiment. The difference between our laboratory and field studies may be due to the chemical and structural variation of surrounding vegetation and to abiotic factors and stressors in the field ('noise'), and the absence of this 'noise' and spatial heterogeneity under laboratory conditions (Gols, 2014; Harvey et al., 2015). Thus, the behavior and performance of natural enemies under laboratory conditions may not always be useful predictors of multitrophic interactions and natural enemy performance in the field (Gols, 2014; Harvey et al., 2015).

In addition to the effects of plant species and architecture on egg mass and larval parasitism rates in the field, we found significant effects of diet quality on the fitness of both healthy and microsporidian-infected larvae of *E. postvittana* under laboratory conditions. Both healthy and infected leaf-roller larvae had greater survivorship, faster development time, and greater lifetime fecundity of viable eggs on the full artificial diet compared to most of the other diets. The

intrinsic rate of increase of healthy larvae was on average 70% higher on full artificial diet and leaves of apple, manzanita and plantain, than on partial artificial diet and leaves of strawberry and Australian tea tree. The intrinsic rate of increase for healthy *E. postvittana* larvae reared on strawberry and Australian tea tree was similar to that of larvae reared on partial artificial diet and due to a reduction in the lifetime fecundity of viable eggs. This suggests that these two plant species did not support as much larval growth as the other plant diets either through lower nutritional quality or greater chemical defenses.

For larvae infected by the microsporidian pathogen, the intrinsic rates of increase were lower on all plant diets compared to full and partial artificial diet and were notably lower for manzanita and strawberry compared to the other plant diets, due to very limited juvenile survivorship and lifetime fecundity of viable eggs. The intrinsic rates of increase of infected larvae reared on manzanita and strawberry were substantially lower than those of healthy larvae reared on these same host plants, suggesting a synergistic interaction between diet and microsporidian infection. It is well known that diet and chemical defenses can stress insect herbivores and decrease their resistance to pathogens (Cory and Hoover, 2006), thereby compounding the effects of infection and low quality diet. For example, viral-infected *Spodoptera exigua* (Hübner) larvae experienced a significant decrease in survivorship when fed on artificial diet with increasing concentrations of leaf resin extract, while non-infected larvae were not significantly affected by leaf resin concentration in the diet (Stubblebine and Langenheim, 1977). In a related study Shikano et al. (2010) demonstrated that viral-infected cabbage looper, *Trichoplusia ni* (Hübner) larvae had lower growth, survival and condition (hemolyph protein concentration) when reared on cucumber compared to broccoli. The number of hemocytes was also significantly lower in infected larvae reared on cucumber, indicating a decreased immune response compared to infected larvae reared on broccoli.

In contrast to larvae reared on manzanita and strawberry, there was much less of a difference between the intrinsic rates of infected and healthy larvae reared on both full and partial artificial diet. As partial artificial diet was included as a nutritional stress treatment devoid of chemical defenses, this suggests that the synergistic effects observed between infection and diet for manzanita and strawberry were more likely to be due to stress effects of chemical defenses than nutritional quality. Previous studies have found extractable phenols and tannins in manzanitas (Kraus et al., 2004), and phenols, flavonoids, flavonols, sulfur compounds, saponins, cyanogenic glycosides and glucosinolates in strawberry leaves (Amil-Ruiz et al., 2011). Differences in the intrinsic rates of increase of infected and healthy larvae reared on apple, Australian tea tree and plantain were intermediate and thus it is less clear whether there were any synergistic interactions between diet and infection for these plant diets. This may well be due to the variation in the classes of chemical defenses present in the plants. For example, the chemical defenses of Australian tea tree include compounds with antimicrobial properties (Brophy et al., 1999), that may have provided some protection against the microsporidian in infected *E. postvittana* larvae.

Although our study included novel as well as coevolved trophic interactions, our results provide little evidence of consistent effects of either host plant origin or domestication (crop versus non-crop) on the fitness of *E. postvittana*. For example, in the field, similar larval parasitism rates were found on apple (exotic crop) and manzanita (native non-crop). The nutritional quality of

these two host plants for *E. postvittana* also appeared to be very similar under laboratory conditions, as shown from the intrinsic rates of increase of healthy larvae ($r_{apple} = 0.14$, $r_{manzanita} = 0.13$). In addition, we found similar field parasitism rates for both eggs and larvae of *E. postvittana* on plantain (exotic non-crop) and on strawberry (native crop) and intrinsic rates of increase of healthy larvae were comparable ($r_{plantain} = 0.13$, $r_{strawberry} = 0.10$) in the laboratory. Although infochemical signals can be disrupted in some domesticated versus traditional host plant varieties (Desurmont et al., 2014), the comparable larval parasitism rates for crop and non-crop plants in this study suggests that, between plant species, domestication had little effect on plant signaling in response to herbivory by *E. postvittana* as a generalist herbivore. Also, the lack of effect of host plant origin on the fitness of *E. postvittana* in this study was surprising as it contrasts with earlier observations that found facilitation between exotic host plants and *E. postvittana* (Engelkes and Mills, 2013).

In the context of the three key hypotheses for tritrophic interactions outlined by Mooney et al. (2012), our study does not address the physiological efficiency of specialist herbivores, but it does provide some additional insights with respect to enemy-free space and enemy exposure. As an exotic generalist herbivore in California, we have demonstrated that, as expected, there was little evidence of enemy-free space for *E. postvittana*, but that the degree of protection from egg and larval parasitism varied among host plants. In contrast, although host plant quality for *E. postvittana* (as estimated from the intrinsic rate of increase of healthy larvae) declined from apple to manzanita and Australian tea tree, and was associated with greater larval development times (exposure), there was no difference in larval parasitism rates in the field. In addition, there did not appear to be a consistent link between host plant quality and the likelihood of synergistic interactions between microsporidian infection and diet. While there are always exceptions to general patterns in ecology, it seems that the lack of host plant quality effects on parasitism and pathogen infection for *E. postvittana* stems from the inclusion of novel interactions, resulting in less predictable consequences (Cox, 2004; Tanaka et al., 2007; Fortuna et al., 2013; Chabaane et al., 2015) and from examining these interactions in a natural study system, which can often be characterized by the 'noise' of many abiotic and biotic factors (Harvey et al., 2015).

4.6 TABLES

Table 1. Effect of plant species, plant architecture, and climate on the probability of parasitism of *Epiphyas postvittana* egg masses by *Trichogramma fasciatum* under field conditions.

Explanatory variables	**N**	**GLMM (individual plants)**	**Statistic**	**d.f.**	***P***
Proportion of egg masses parasitized	209	binomial	χ^2		
Plant species			110.38	8	**< 0.001**
		GLM (block means)			
Proportion of egg masses parasitized	36	quasibinomial	χ^2		
Plant height			21.28	1	**< 0.001**
Plant width			0.01	1	0.91
No. of branches			1.92	1	0.14
No. of leaves			0.53	1	0.44
Leaf surface area			3.86	1	**0.05**
Trichome density			0.52	1	0.81
Temperature			2.65	1	0.09
Wind speed			0.42	1	0.49

Table 2. Effect of plant species, plant architecture, and climate on the probability of larval parasitism of *Epiphyas postvittana* by resident larval parasitoids (*Meteorus ictericus* and *Enytus eureka*) under field conditions.

Explanatory variables	**N**	**GLMM (individual plants)**	**Statistic**	**d.f.**	***P***
Percent parasitized larvae	159	binomial	χ^2		
Plant species			40.08	8	**< 0.001**
		GLM (block means)			
Percent parasitized larvae	41	binomial	χ^2		
Plant height			6.81	1	**0.01**
Plant width			3.60	1	0.07
No. of branches			0.98	1	0.31
No. of leaves			0.00	1	0.97
Leaf surface area			2.21	1	0.13
Trichome density			0.08	1	0.78
Temperature*Wind speed			5.31	1	**0.02**

Table 3. Effect of plant species, leaf characteristics and parasitoid size on the success of contact and search time of *Trichogramma fasciatum* foraging for egg masses of *Epiphyas postvittana* under laboratory conditions.

Explanatory variables	**N**	**GLM**	**Statistic**	**d.f.**	***P***
Success of contact	118	binomial	$\chi 2$		
Plant species			9.65	1	0.29
No. of leaves			0.00	1	0.99
Total leaf surface area			3.34	1	0.07
Host distance			0.02	1	0.88
Parasitoid size			1.48	1	0.22
Search time	66	quasi-Poisson	*F*		
Plant species			2.5	8, 52	**0.02**
No. of leaves			0.02	1, 48	0.90
Total leaf surface area			0.04	1, 49	0.84
Host distance			0.67	1, 50	0.42
Parasitoid size			2.32	1, 51	0.13

Table 4. Effect of leaf surface area and trichome density of *Plantago lanceolata* and parasitoid size on the success of contact and search time of *Trichogramma fasciatum* foraging for egg masses of *Epiphyas postvittana* under laboratory conditions.

Explanatory variables	**N**	**GLM**	**Statistic**	**d.f.**	***P***
Success of contact	22	binomial	$\chi 2$		
Trichome density			6.34	1	**0.01**
Leaf surface area			3.62	1	**0.06**
Host distance			0.04	1	0.84
Parasitoid size			0.46	1	0.50
Host search time	14	quasi-Poisson	*F*		
Trichome density			1.21	1, 11	0.29
Leaf surface area			0.00	1, 10	0.99
Host distance			1.81	1, 12	0.20
Parasitoid size			0.01	1, 9	0.91

Table 5. Effect of infection by *Nosema fumiferanae* and diet on the life history performance of *Epiphyas postvittana*. N is the number of control (C) and infected (I) replicate rearing cups (with five initial larvae per cup).

Explanatory variables	**N (C, I)**	**GLM**	**Statistic**	**d.f.**	***P***
Juvenile survivorship	72, 72	binomial	$\chi 2$		
Infection status			172.43	1	**< 0.001**
Diet			14.70	6	**0.02**
Juvenile development time	67, 21	Poisson	$\chi 2$		
Infection status			0.21	1	0.64
Diet			67.16	6	**< 0.001**
Gender			7.00	1	**0.01**
Lifetime fecundity (viable eggs)	50, 10	Poisson	$\chi 2$		
Infection status*Diet			491.26	5	**< 0.001**

Table S4.1. Vital rates for *Epiphyas postvittana* fed on different diets (full and partial are artificial diets) and either healthy (non-infected) or infected by *Nosema fumiferanae*. Data are represented as grand means as larvae fed in groups of five on the seven diets. Development time (DT, days ± SE) and adult longevity (days ± SE) are based on the grand means from females.

Treatment	Diet	Larval Survivorship	Larva DT (Days ± SE)	Pupal Survivorship	Pupa DT (Days ± SE)	Female sex-ratio	Reproductive success	Pre-Reproductive (Days ± SE)	Longevity (Days ± SE)	Lifetime Fecundity (Eggs ± SE)	Viable offspring
Healthy	Full	78%	23.5 ± 1.1	85%	9.3 ± 0.3	51%	93%	5.1 ± 1.0	17.5 ± 1.6	452.3 ± 125.3	74%
	Partial	70%	31.8 ± 2.0	76%	8.6 ± 0.3	53%	88%	4.1 ± 0.7	13.3 ± 1.3	178.4 ± 30.8	63%
	Apple	63%	23.7 ± 1.2	80%	8.2 ± 0.2	47%	100%	2.5 ± 0.3	15.4 ± 1.8	410.6 ± 76.1	72%
	Manzanita	65%	33.7 ± 1.1	81%	8.4 ± 0.5	55%	93%	3.4 ± 0.4	15.8 ± 1.5	403.9 ± 27.2	79%
	Plantain	55%	30.3 ± 3.1	72%	8.3 ± 0.1	53%	93%	4.6 ± 0.9	15.2 ± 1.2	447.1 ± 75.8	79%
	Strawberry	72%	31.6 ± 1.5	72%	8.4 ± 0.2	42%	83%	3.7 ± 1.0	12.7 ± 1.5	271.0 ± 44.3	71%
	Tea Tree	44%	42.3 ± 3.9	76%	8.3 ± 0.2	60%	80%	4.6 ± 1.0	13.4 ± 0.4	202.7 ± 30.0	65%
Infected	Full	71%	28.2 ± 2.7	26%	7.8 ± 0.4	34%	50%	2.5	10.0 ± 3.0	415.5	79%
	Partial	20%	36.8 ± 4.0	30%	9.3 ± 0.9	56%	100%	2.5 ± 0.5	13.7 ± 2.9	174.5 ± 54.5	48%
	Apple	22%	28. ± 2.1	27%	8.0 ± 0.6	36%	67%	4.0 ± 2.0	12.0 ± 3.5	254.5 ± 111.5	47%
	Manzanita	6%	31.0 ± 1.0	67%	8.0	67%	100%	10.0	26.0	64.0	13%
	Plantain	24%	26.1 ± 2.6	29%	8.5 ± 0.5	46%	75%	5.3 ± 0.3	21.7 ± 4.1	235.7 ± 118.1	37%
	Strawberry	8%	37.0	0%	NA	33%	NA	NA	NA	NA	NA
	Tea Tree	10%	45.7 ± 7.6	60%	9.0 ± 1.0	60%	50%	6.0	13.0 ± 0.0	132.0	39%

4.7 FIGURES

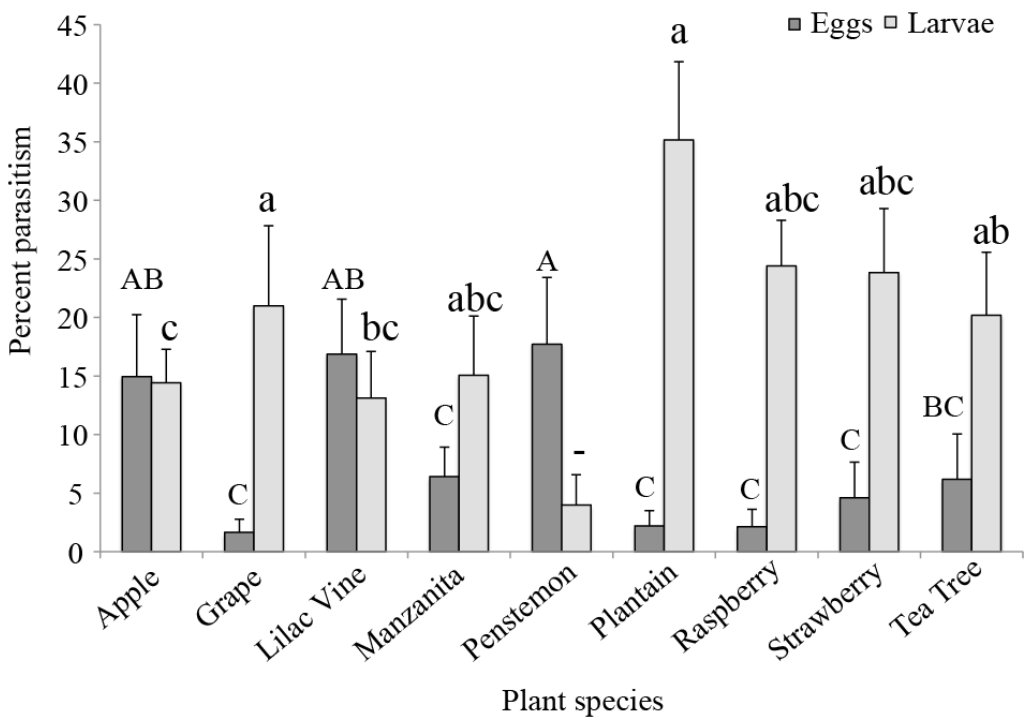

Figure 1. The influence of host plants on mean percent parasitism of egg masses (dark grey) and larvae (light grey) of *Epiphyas postvittana* by resident parasitoid wasps in a common garden experiment in Daly City, CA. Letters represent significant differences in percent parasitism among host plants (larval parasitism on penstemon was excluded from multiple comparison analysis due to insufficient block replication).

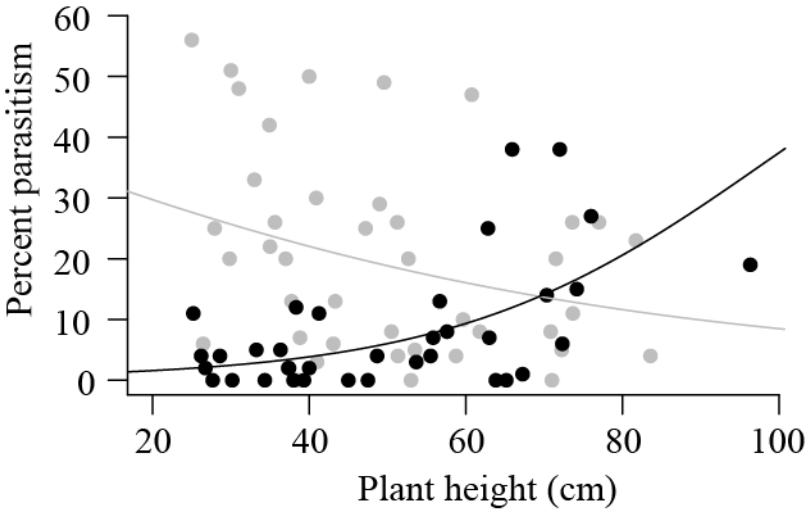

Figure 2. The mean percent parasitism of egg masses (black) and larvae (grey) of *Epiphyas postvittana* by resident parasitoid wasps in relation to plant height in a common garden experiment in Daly City, CA; $y_{eggs} = 1/(1+\exp(4.93 - 0.04x))$, $y_{larvae} = 1/(1+\exp(0.45 + 0.02x))$.

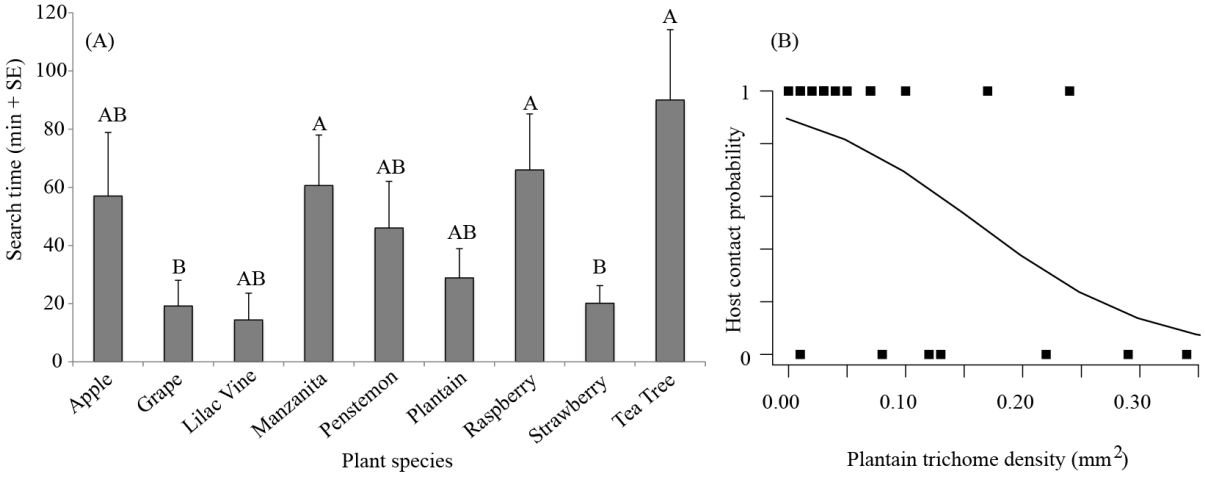

Figure 3. The mean search time (A) for host egg masses of *Epiphyas postvittana* by *Trichogramma fasciatum* on different host plant species in the laboratory with letters representing significant differences between host plants, and the probability of contacting an egg mass (B) in relation to the density of trichomes on leaves of *Plantago lanceolata*, $y = 1/(1+\exp(-2.16+13.30x))$.

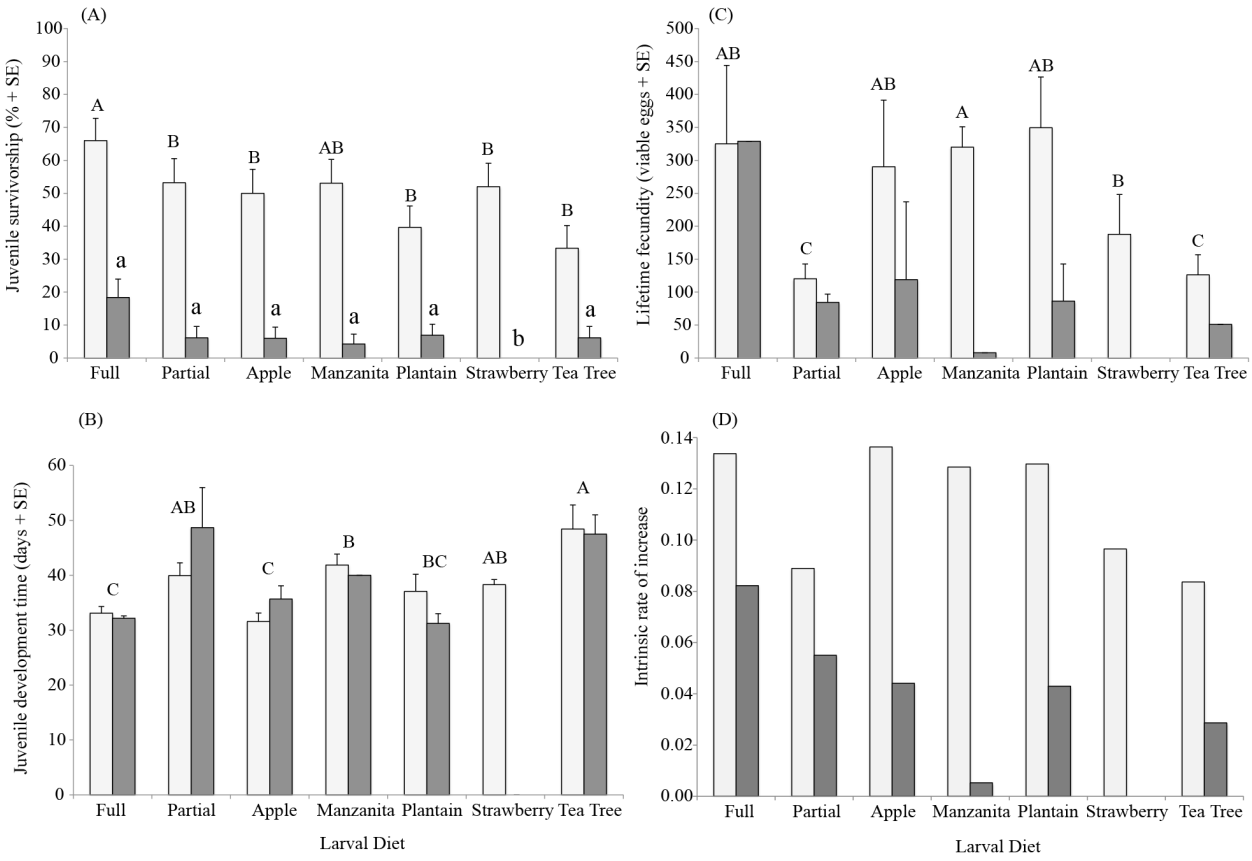

Figure 4. Mean juvenile survivorship (A) and female juvenile development time (B), per capita lifetime fecundity of viable eggs (C) and intrinsic rate of increase (D) for *Epiphyas postvittana* fed on different diets (full and partial are artificial diets) and either infected by *Nosema fumiferanae* (dark grey) or non-infected controls (light grey). Data are represented as grand means as larvae fed in groups of five on the seven diets. Letters represent significant differences among infection status and/or diet.

CONCLUSION

In an era of global warming, ecological communities are increasingly influenced by species invasions and shifting species ranges (Sala et al., 2000; Tylianakis et al., 2008; Sorte et al., 2010). To predict and mitigate potential negative impacts, it is important to understand how interactions with natural enemies can affect the distribution and abundance of species in motion. Shifting geographic ranges may allow hosts to escape from natural enemies (Keane and Crawley, 2002; Mitchell and Power, 2003; Torchin et al., 2003; Hopper et al., 2014), transport coevolved parasites (Strauss et al., 2012), or experience biotic resistance from novel resident enemies in their new range (Elton, 1958; Maron and Vila, 2001; Levine et al., 2004; Carlsson et al., 2011; Dumont et al., 2011; Kimbro et al., 2013; Alofs and Jackson, 2014). Additionally, whether the losses or gains of enemies during a range shift will affect the population abundance of a species in motion will depend on the climatic tolerances of all of the interacting species (Gutierrez et al., 2008). The exotic light brown apple moth, *Epiphyas postvittana,* provides a unique opportunity to examine multitrophic interactions and potential mechanisms for the suppression of a generalist herbivore in its novel range. Although this highly polyphagous exotic leafroller continues to increase its range in coastal California, an initial increase in abundance in San Francisco and Santa Cruz has been followed by a subsequent decline (Bürgi and Mills, 2014).

In Chapter 1, I investigated the occurrence and fitness consequences of infanticide for *Goniozus jacintae,* a potential biological control agent of *E. postvittana*. *G. jacintae* has a fecundity of 64 eggs, a minimal level of brood guarding, and readily commits both ovicide and larvicide. Based on an evolutionary model of infanticide, Strand and Godfray (1989) suggested that ovicide is more likely to occur in parasitoids when: (1) the first clutch has a competitive advantage for superparasitized hosts; (2) non-parasitized hosts are rare; (3) time taken to perform ovicide is minimal; and (4) the risk of removing an individual's own clutch is low. My analysis of macronutrient concentrations in relation to the fitness consequences of infanticide is the first to provide evidence that improved nutritional quality could be an important benefit of infanticide for a gregarious ectoparasitoid, and one that may be responsible for the larger clutch size and enhanced survivorship observed among secondary ovicidal clutches of *G. jacintae*. Although classical biological control of *E. postvittana* is not currently being pursued in California, this study contributes to our understanding of the evolution of infanticide as a response to intraspecific competition among female parasitoid wasps. Whether infanticide is a beneficial or detrimental characteristic for a parasitoid selected for use in a classical biological control program has never been addressed. For a parasitoid such as *G. jacintae,* to commit infanticide when non-parasitized hosts are rare could be considered to be a beneficial trait for biological control provided that it leads to the production of a greater number of progeny with greater survivorship. This could help a parasitoid to maintain slightly higher population densities at times of low host abundance and consequently a more rapid response to an increase in host density. In this context it would be interesting to compare bethylid parasitoids, that do exhibit infanticide, to that of gregarious braconid ectoparasitoids, that do not, in the historical record of classical biological control successes.

During the course of my dissertation, I discovered that both a laboratory colony and field populations of *E. postvittana* were infected by a microsporidium. Due to the potential significance of microsporidia in populations of *E. postvittana,* my aim in Chapter 2 was to

identify and determine the origin of the microsporidium. From the pathology, morphology and sequence results from molecular markers, I was able to place this isolate as a subspecies in the *Nosema fumiferanae* species complex (*N. fumiferanae postvittana* subsp. n.) that also includes *Nosema* sp. CO and *Nosema* sp. CPP. Thus, *N. fumiferanae postvittana* appears to be an indigenous species originating from resident tortricid hosts, and sharing a very close evolutionary relationship with other *N. fumiferanae* species isolated from North America forest habitats. However, I cannot exclude the possibility that *N. fumiferanae postvittana* was carried by *E. postvittana* when it invaded California, as no studies of infection of *E. postvittana* by microsporidia in Australia or New Zealand have been published. My observations of systemic tissue infection, moderate pathology and vertical transmission of *N. fumiferanae postvittana* from infected adults to their offspring, also support the notion that *Nosema* species are low virulence pathogens that develop slowly, that allow their hosts to continue to develop through to the adult stage, and that are maintained in the host population by vertical transmission (Anderson and May, 1981; Solter, 2006).

To confirm whether *N. fumiferanae postvittana* is in fact an indigenous species in North America, future studies should survey *E. postvittana* populations in Australia and New Zealand to screen them for Microsporidia and identify local isolates in the region of origin. If the *N. fumiferanae* species complex can be confirmed to be of North American origin, the acquisition of an indigenous microsporidian by *E. postvittana* as a competent exotic host may have been facilitated by parasitoids that are shared with native leafrollers. This then could generate the potential for parasite spillback, a form of apparent competition in which infection of a novel exotic host by a native parasite can spillback to cause greater levels of infection of native hosts (Kelly et al. 2009).

In Chapter 3, I conducted laboratory experiments and field sampling to understand the significance of the novel pathogen-host relationship between *N. fumiferanae postvittana* and *E. postvittana*. From the dose-response experiments under laboratory conditions, there were notable fitness consequences for infection of *E. postvittana* larvae by *N. fumiferanae postvittana*. Comparing healthy larvae to larvae infected with up to 10^5 spores, I found a reduction in juvenile survivorship (from 100% to 26%), a prolongation of juvenile development time (increase of 9 to 10 days for females and males respectively), a reduction in viable lifetime fecundity (from 788 to 1) and a reduction in the intrinsic rate of natural increase (from 0.18 to 0.008). I estimated the LD_{50} to be 1.8×10^4 spores, comparable to that for *N. fumiferanae* in fourth instar host larvae of the spruce budworm, *C. fumiferana* (Bauer and Nordin, 1988). At low doses of spores ingested, the effects of *N. fumiferanae postvittana* were more chronic than acute, and had a greater impact on the viable lifetime fecundity of females than on other life history parameters. From our stage-structured matrix models, I determined that these sublethal effects could still have an important effect on the intrinsic rate of natural increase of *E. postvittana,* particularly for infections that were initiated with 10^3 spores or more. The latent period for *E. postvittana* infected with *N. fumiferanae postvittana* was 12.7 days when second instar larvae were infected with 10^3 spores. This relatively short latent period, combined with the dose-dependent sublethal and lethal effects of *N. fumiferanae postvittana* suggest that it could have a similar effect on field populations of *E. postvittana* as the related isolate, *N. fumiferanae,* which can achieve over 80% infection in populations of *C. fumiferana* (Thomson, 1960a).

The field sampling demonstrated that population densities of *E. postvittana* varied with location, season and plant species, and that population densities in San Francisco and Santa Cruz in 2013/14 had further declined from those documented in the same two locations from 2009 to 2012 (Bürgi et al., 2015). Using qPCR, I detected the microsporidium in hosts from all locations, seasons and plant species. Prevalence was low (5% overall) and accompanied by a low intensity of infection (mean of 226 spores). Prevalence did not appear to be influenced consistently by host density or by season.

Although our laboratory study demonstrated strong negative effects of the microsporidium, *N. fumiferanae postvittana* on the life-history performance of *E. postvittana*, combined with a relatively short latent period, indicating the potential for host population suppression, I did not find any evidence for strong impacts in the field. The low levels of infection by *N. fumiferanae postvittana* in the field contrasts with the greater than expected levels of parasitism by resident parasitoid wasps on this exotic host species (Bürgi et al., 2015). Nonetheless, as discussed by Levine et al. (2004), although resident natural enemies are unlikely to repel invasions completely, these biotic interactions, including that of *N. fumiferanae postvittana*, can still be of importance in reducing the abundance and spread of exotic species, such as *E. postvittana*.

As neither parasitoids nor the microsporidian pathogen appear to act as sole mechanisms for the decline of *E. postvittana,* I examined multitrophic interactions and the effects of host plants on enemy-free space for *E. postvittana* in Chapter 4. As this exotic generalist herbivore feeds on plants from a wide range of families, particular host plants may have an important influence on its performance and potential for enemy-free space with respect to resident parasitoids and pathogens. Based on a common garden experiment conducted in the field, I found that parasitism of egg masses varied by a factor of 4.7 between host plant species. Plant architecture influenced the extent of egg parasitism with a higher risk of parasitism on taller plants than shorter plants. Larval parasitism also varied among plant species by a factor of 2.1 with a higher risk of parasitism for larvae on shorter plants than taller plants. In the laboratory, I conducted experiments to further examine which aspects of plant architecture and leaf morphology may have influenced parasitism rates for egg masses in the field. Time taken for a parasitoid to find an egg mass varied among plant species, but did not correspond to rates of egg mass parasitism in the field. In addition, the probability of contacting an egg mass on leaves of *Plantago lanceolata* decreased with trichome density. In the laboratory, I also found significant effects of diet on the fitness of both healthy and microsporidian-infected *E. postvittana*, with evidence of synergistic effects between diet and infection for some of the plants tested.

Overall, there was a significant effect of host plant on the extent of enemy-free space for *E. postvittana*. As an exotic generalist herbivore in California, I have demonstrated that, as expected, there was little evidence of enemy-free space for *E. postvittana*, but that the degree of protection from egg and larval parasitism varied among host plants. However, although host plant quality for *E. postvittana* (as estimated from the intrinsic rate of natural increase of healthy larvae) declined from apple to manzanita and Australian tea tree, and was associated with greater larval development times (exposure), there was no difference in larval parasitism rates in the field associated with host plant quality. In addition, there did not appear to be a consistent link between host plant quality and the likelihood of synergistic interactions between microsporidian infection and diet. While there are always exceptions to general patterns in ecology, it seems that

the lack of host plant quality effects on pathogen infection and parasitism for *E. postvittana* stems from the inclusion of novel interactions with less predictable consequences (Cox, 2004; Tanaka et al., 2007; Fortuna et al., 2013; Chabaane et al., 2015) and from the added 'noise' that is characteristic of natural study systems (Harvey et al., 2015).

As I did not conduct behavioral assays in my study of multitrophic interactions, future studies should include choice-tests to determine whether *E. postvittana* prefers certain host plants to others, and investigation of whether preferred host plants translate to enhanced enemy-free space in the laboratory and in the field. It would also be interesting to determine the bottom-up effects of abiotic stressors, such as drought or nutrient limitation, on multitrophic interactions and the fitness consequences of microsporidian infection for *E. postvittana*. Significant effects of climatic stress have been demonstrated in bi-trophic interactions between hosts and their pathogens (Altizer et al., 2013), but the effects of climatic stress on multitrophic interactions involving pathogens have yet to be examined.

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