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Transmissibility of *Nosema lymantriae* spores in *Lymantria dispar* infected with *Bacillus thuringiensis*

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Kurzfassung

Übertragbarkeit von *Nosema lymantriae* Sporen bei zeitgleicher Infektion von *Lymantria dispar* mit *Bacillus thuringiensis*

Das Verständnis der komplexen Interaktionen zwischen mehreren Organismen, wenn diese zusammen in Programmen der biologischen Schädlingsbekämpfung eingesetzt werden, ist bedeutend für die Beurteilung ihrer potentiellen Wirksamkeit. *Bacillus thuringiensis* var. *kurstaki* (Bt) ist ein bekanntes mikrobiologisches Pestizid, welches für die Bekämpfung von *Lymantria dispar*, dem Schwammspinner, einem bedeutenden heimischen und in Nordamerika invasiven Forstschädling mit potentieller Gefahr für Obstbäume und Korkeichenwälder, genutzt wird. *Nosema lymantriae* ist eine heimische entomopathogene Mikrosporidienart des Schwammspinners, die ebenfalls Potenzial für den Einsatz in der biologischen Schädlingsbekämpfung zeigt. *Nosema lymantriae* wird horizontal über Kot und Kadaver übertragen, vertikal über transovariell infizierte Eier. Die vorliegende Arbeit untersuchte den Einfluss sublethaler Bt-Konzentrationen auf die Menge von Mikrosporidiensporen im Kot von Schwammspinnerlarven, wenn diese zeitgleich mit beiden Pathogenen infiziert waren, und deren Infektiosität sowie Effekte auf Schlupf, Überleben und Infektion von Larven, wenn deren Eltern mit beiden Pathogenen infiziert waren. Sublethale Konzentrationen von Bt (0.5 IU, 1 IU, 2 IU) beeinflussten nicht die durchschnittliche Sporenzahl im Kot, der während des 4. und 5. Larvenstadiums gesammelt wurde. Die Gesamtmenge der Sporen im Kot variierte zwischen 1.0 und 3.3×10^7 Sporen während des Erfassungszeitraumes. Auf Grund unerwartet geringer Infektionsraten dieser Sporen (< 35%) kann der Einfluss von Bt auf die Infektiosität von *N. lymantriae* nicht eingeschätzt werden. Kombinierte Infektionen beeinträchtigten nicht den Schlupf und das Überleben der Nachkommen. Diese Daten deuten daher darauf hin, dass sublethale Dosen von Bt keine negative Effekte auf die Übertragbarkeit von *N. lymantriae* haben.

Schlagwörter: *Lymantria dispar, Nosema lymantriae*, *Bacillus thuringiensis* subsp. *kurstaki*, horizontale Übertragung, mikrobielle Schädlingskontrolle

Abstract

Understanding the complex interactions of multiple organisms often involved in biological control methods is critical for evaluating their potential effectiveness. *Bacillus thuringiensis* var. *kurstaki* (Bt) is a common microbial pesticide used to control *Lymantria dispar* (gypsy moth), a major forest defoliator native to Eurasia and invasive in North America, and a potential threat to fruit tree and cork oak orchards. *Nosema lymantriae* is a native microsporidian entomopathogen of gypsy moth that shows potential as a biological control agent. *N. lymantriae* can spread horizontally via contaminated feces and cadavers, and vertically via transovarially-infected eggs. This study examined the impact of sublethal Bt doses on the amount of microsporidian spores in feces of gypsy moth simultaneously infected with both pathogens, as well as the impact of Bt on the subsequent infectivity of spores, and effects on hatching, survival, and infectivity of larvae derived from mixed-infected parents. Three sublethal doses of Bt (0.5 IU, 1 IU, and 2 IU) were found not to significantly affect mean spore number in feces collected during the fourth and fifth instars. Total spore production in feces during the collection period ranged from 1.0-3.3 x 10^7 spores. The impact of sublethal Bt doses on subsequent infectivity of collected *N. lymantriae* spores was not elucidated due to unexpectedly low infection rates among all treatments (< 35%). Mixed infections also did not conspicuously affect larval hatching and survivability. These data therefore suggest that sublethal doses of Bt have no negative effect on transmissibility of *N. lymantriae.*

Keywords: *Lymantria dispar*, *Nosema lymantriae*, *Bacillus thuringiensis* subsp. *kurstak*i, horizontal transmission, microbial control.

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1 Introduction

Biological control agents represent an increasingly important alternative to chemical insecticides for controlling agricultural pests (Thomas and Willis 1998, Barbosa 1998). Due to the involvement of multiple organisms, biocontrol methods can have more complex effects and interactions than traditional pest control methods (Rice-Mahr *et al.* 2001). Numerous studies have examined insect-pathogen interactions in the context of biological control, but less emphasis has been placed on the interactions of multiple or mixed infections by biocontrol pathogens in the target host insect (Thomas *et al.* 2003). Mixed infections can exhibit several kinds of interactions among each other within their shared host. Interactions can be antagonistic, where one pathogen suppresses another, or pathogens suppress each other. Pathogens may also exhibit synergistic or additive interactions, where mixed infection enhances the infectivity or virulence of other pathogens within the host (Thomas *et al.* 2003). Understanding these interactions is critical for evaluating the potential efficacy of using multiple pathogens in biological control schemes as well the possible effects on host population dynamics of introduced pathogens on native pathogens. This study examined aspects of mixed infection of a natural microsporidian pathogen with the microbial pesticide *Bacillus thuringiensis* (Bt) in the forest pest *Lymantria dispar* (gypsy moth).

The gypsy moth, *Lymantria dispar* L. (Lepidoptera: Erebidae: Lymantriinae, Fauna Europaea 2012) is a major forest defoliator native to the temperate forests of Eurasia that has become an invasive pest in North America (Liebhold 2003). The gypsy moth's life cycle begins when larvae hatch from densely hair-covered egg masses in early- to mid-spring, often concurrently with budding of hardwood host trees (Leonard 1981, McManus *et al.* 1989). Newly hatched larvae are phototropic and therefore move towards the tree crown to access foliage and to facilitate wind dispersal by "ballooning" on threads of silk (Leonard 1981, McManus *et al.* 1989).

Larvae typically undergo four (male) to five (female) molts (five or six instars), resulting from a considerable increase in foliage feeding and a concomitant, thousand-fold increase in weight and thirty-fold increase in length (Leonard 1981). Foliage feeding behavior varies by both instar and larval density. Through the third instar, larvae feed during the day, with peek feeding times in the early morning and late afternoon, after which larvae secure themselves around abaxial leaf midribs (Leonard 1981) near feeding sites in the crowns of host trees (McManus *et al.* 1989). After molting into the fourth instar, larvae at low densities feed primarily at night, and rest during the day by moving away from the crown to find protected crevices in bark and branches. At high densities, however, late instar larvae can feed continuously, day and night (McManus *et al.* 1989). Nielsen and Hajek (2006) observed that the fungal entomopathogen *Entomophaga maimaiga* manipulates these day-night behavior patterns to enhance spore transmission.

Larvae pupate in late-June to early July, and adults emerge 7-17 days later (Leonard 1981, McManus *et al.*1989). Adult moths exhibit considerable sexual dimorphism. Females have massive, egg-filled abdomens, white wings, and in European and North American populations, females cannot fly, while males have mottled-brown wings and plumose antennae (Leonard 1981). The stationary females attract males by means of pheromones. Once mating occurs, eggs are usually laid within 24 hours (Leonard 1981). Larvae develop in the eggs four to six

weeks later, followed by an extended diapause over winter until hatching in spring (McManus *et al.* 1989).

Gypsy moth can potentially feed on more than 500 host tree species (Liebhold *et al.* 1995), preferring primarily *Quercus* and *Populus* spp., though other hardwoods such as *Tilia*, *Salix*, and *Alnus* spp. are also common hosts (McManus *et al.* 1989). Late instar larvae are less particular about hosts, and have been found feeding on coniferous species (Leonard 1981). The consequences of gypsy moth defoliation on hosts vary among species, on the extent and number of defoliations, as well as the presence and virulence of secondary tree pathogens (Davidson *et al.* 1999). Oaks and other hardwoods can survive multiple, complete, defoliations, whereas coniferous species have much higher rates of mortality after a single, extensive defoliation (Davidson *et al.* 2001, Davidson *et al.* 1999, McManus *et al.* 1989).

The gypsy moth was accidentally introduced to North America in 1869 by E. L Trouvelot, who had been raising larvae at his Massachusetts home, possibly for evaluating its potential use in commercial silk production (Liebhold 2003). Gypsy moth outbreaks were occurring across eastern Massachusetts in the 1890s, and by spread to most of New England by the 1930s, followed by eastern New York in the 1960s. By the 1990s, outbreaks had been documented in the Mid-Atlantic States, Virginia, West Virginia, Pennsylvania, Michigan, and Ontario. As of 2008, gypsy moth had spread further west into Ohio, Indiana, northeast Illinois, Wisconsin, and the Upper Peninsula of Michigan (Liebhold 2003). The present distribution represents only a quarter of gypsy moth's potential range in North America (Liebhold 2000) and is expanding at 21 km/year (Liebhold 2003). Gypsy moth outbreaks can also threaten fruit tree orchards (Global Invasive Species Database 2012) and have been a significant and persistent pest to cork oak orchards in Sardinia (Vogiatzakis *et al.* 2005, Luciano and Prota 1979).

Gypsy moth populations usually persist at low densities, followed by rapid increases in populations over several generations that lead to a full outbreak (Elkinton and Liebhold 1990). In Europe, outbreaks are most frequent in the southern and southeastern parts of the continent, as well as in areas further north with recent warm and dry weather patterns (Hoch *et al.* 2001) while in North America, outbreaks are often in forests near urban areas (McManus and McIntyre 1981). Globally, outbreaks appear to be in approximately 8-12 year cycles (Johnson *et al.* 2005, McManus and McIntyre 1981). Outbreaks can occur synchronously among populations within a given continent, but have not been observed to occur simultaneously among global populations (Johnson *et al.* 2005).

Historically, authorities used several classes of insecticides to treat gypsy moth outbreaks. Paris Green, and later lead-arsenite, predominated from the end of the 19th century until the Second World War. DDT (Dichloro-diphenyl-trichloroethane) was used extensively after the War and was highly effective until public opposition to DDT's notorious non-target effects led to its eventual ban, replaced by carbaryl and later trichlorfon in the 1960s and 1970s (Liebhold and McManus 1999, White *et al.*1981). Acephates and the molt-interfering diflubenzuron were approved for use on gypsy moth in the late 1970s, though the latter was preferred by the 1980s due to its less detrimental non-target effects (Liebhold and McManus 1999, White *et al.* 1981).

Yet diflubenzuron still affects a diverse array of non-target arthropods (Goertz *et al.* 2004) and is particularly harmful to freshwater invertebrates (Liebhold and McManus 1999).

More than 60 species of natural enemies have been introduced to try to control North American gypsy moth populations (Kenis and Vaamonde 1998). Egg, larval, and pupal parasitoids that include taxa of Encyrtidae, Braconidae, Ichneumonidae, Torymidae and Chalcididae wasps and Tachinidae flies have been introduced, but only 11 have become established. Most have failed due to lack of alternate hosts (Kenis and Vaamonde 1998). Established parasitoids do not appear to effect North American gypsy moth populations in a density-dependent manner (Liebhold *et al.* 2000) and have not affected the spread of gypsy moth on the continent.

Pathogenic natural enemies can control high-density (outbreak) gypsy moth populations more effectively than parasitoids, but still have limited or non-existent commercial applications. The nucleopolyhedrosis virus (NPV) is an epizootic, highly host specific pathogen that causes wilt disease in gypsy moth larvae. NPV spreads horizontally when rotting cadavers killed by the virus contaminate foliage, as well as by transovum (vertical) transmission (Reardon *et al.* 2003, Lewis 1981). In its native European range, NPV can be epizootic during the end of gypsy moth outbreak phases and similar density-dependent declines in parts of North America (McManus and Solter 2003, Elkinton and Liebhold 1990). Commercial formulations NPV are now available as *Gypchek,* but its *in vivo* production methods make it an expensive option for treatment of gypsy moth outbreaks (Liebhold 2003, Reardon *et al.* 2003).

Entomophaga maimaiga is a fungal pathogen introduced to North America from Japan in the early 20th century that caused an unexpected, epizootic decline in New England gypsy moth populations in 1989 and Virginia in 1992 (Liebhold 2003, Hajek 1999). *E. maimaiga*'s potential as a biological control agent is still being investigated (Hajek 1999), but Pilarska *et al.* (2006) found *E. maimaiga* introduced in 2000 had become successfully established in several Bulgarian forests by 2004. However, difficult culturing requirements have so far inhibited *E. maimaiga*'s development into a commercially available product (Liebhold 2003, Hajek 1999).

Among the most widespread and effective control method for gypsy moth outbreaks is Bt, a microbial pesticide comprised of the spores and crystal toxins produced by the ubiquitous soil bacterium *Bacillus thuringiensis* (Liebhold and McManus 1999, Reardon *et al.* 1994). *Bacillus thuringiensis* is a flagellated, rod-shaped, Gram-postive bacterium first isolated in silk moth larvae in Japan (1901), and later in flour moth larvae in Germany (1911), and with its experimental application on gypsy moth in New England in the 1920s, Bt became the first modern microbial insecticide (Reardon *et al.* 1994). Commercial Bt formulations became available in France in 1930s and later the United States in the 1950s, but modern formulations used on gypsy moth are derived from the *kurstaki* strain developed in the 1970s, which selectively targets Lepidopteran taxa (Reardon *et al.* 1994). By the 1980s and 1990s, Bt had become a principle control method against gypsy moth outbreaks (Liebhold and McManus 1999, Reardon *et al.* 1994).

Under low-nutrient conditions in nature (Madigan *et al.* 2002), or under induced *in vitro* conditions, *Bacillus thuringiensis* undergoes a process of sporulation, wherein a resistant endospore and an octahedral endotoxin are produced. This octahedral protein complex is non-

toxic until exposed to the alkaline insect midgut, where protelytic enzymes cleave the protein into smaller subunits known as δ-endotoxins or insecticidal crystal proteins (ICPs) (Reardon *et al.* 1994). ICPs bind to midgut cell membranes and create ion-selective channels that disrupt cell osmolality, leading to water uptake and eventual cytolysis. The resulting perforation of the midgut ultimately causes host death primarily by starvation, and secondarily by septicemia from bacterial contamination of the hemolymph (Reardon *et al.* 1994).

Bt sprayed on foliage has a short half-life of twelve to thirty-two hours in the field due to solar UV degradation. However, at typical spray concentrations, the effective insecticidal activity lasts for five to six days (Reardon *et al.* 1994). Bt is therefore not an epizootic microbial pathogen that can become established in environment, but rather a microbial pesticide. The timing of sprays against gypsy moth must balance the amount of foliage surface area versus the developmental stage of larvae, where earlier instar larvae are more susceptible to the Bt toxin (Reardon *et al.* 1994, Dubois 1981). Commonly, this balance occurs when about half the larvae are in the second instar and the remainder comprised of first and third instars (Reardon *et al.* 1994). The Bt strains used against gypsy moth only target other lepidopterans, and extensive studies have demonstrated Bt is non-toxic to immunologically healthy humans and other vertebrates who come into contact with Bt directly or that consume Bt-inoculated larvae (Durkin *et al.* 2004, Siegel 2001, Reardon *et al.* 1994)

Owing to its single mode of action like other insecticides, the evolution of resistance to Bt is possible. Bt-resistant strains of several insect orders, including Lepidopterans (Tabashnik 1994, Ferre *et al.* 1991) have evolved under experimental conditions, particularly with transgenic crops containing Bt-toxin genes (Tabashnik *et al.* 2003). Further, insects have used multiple genes and strategies to evolve resistance to Bt (Heckel *et al.* 2007). Dubois (1993), however, noted that Bt treatment of gypsy moth has several features that might inhibit the evolution of resistance, including limited number of sprays per year, only one gypsy moth generation per year, gene flow among populations and the limited persistence of Bt in the environment, among others (Reardon *et al.* 1994). Nevertheless, the possibility of resistance combined with Bt's toxicity to non-target Lepidopterans suggests other or augmentative treatments for gypsy moth outbreaks should be sought (Liebhold and McManus 1999).

Microsporidia are a possible, and often overlooked, natural enemies and candidates for augmentative and classical biological control of gypsy moth (Hoch *et al.* 2008, Goertz and Hoch 2009). Microsporidia are obligate, intracellular parasites comprising some 1200 species in 150 genera (Keeling and Fast 2002) that infect many animal phyla, though over half of the genera are pathogens of insects (Becnel and Andreadis 1999). Once thought to be basal, primitive, amitochondriate eukaryotes assigned to the Archeazoa, their taxonomic fluctuated with improvements in molecular phylogenetic techniques (Keeling and Fast 2002). Resolving erroneous long-branch attraction placed microsporidia within the fungi, formerly as Zygomycota (Keeling and Fast 2002) and more recently as their own phylum (Hibbett *et al.* 2007). The apparent primitive features of microsporida are now attributed to be a consequence of reduction and selection for their highly specialized, intracellular parasitic habits (Keeling and Fast 2002).

Microsporidia transmit within or among hosts as extracellular spores. The spores are comprised primarily of structures involved in their unique, highly specialized, infection mechanism (Keeling and Fast 2002). Poorly understood *in vivo* signals induce spores to eject harpoon-like polar tubes, piercing host target cells. The injection apparatus simultaneously forces the spore cytoplasm (sporoplasm) through the polar tube, emerging inside the host cytoplasm (Keeling and Fast 2002, Goertz and Hoch 2008a). The intracellular sporoplasm, or meront, is the primary parasitic form that alters and reorganizes host cells optimized for the metabolism (xenomas) and vegetative reproduction (merogony) (Keeling and Fast 2002) that commonly circumvents host immune responses (Becneal and Andreadis 1999, Keeling and Fast 2002). A second mode of reproduction can involve the formation of new spores to infect other hosts or new tissues within the same host. Sporogony exhibits considerable diversity and complexity among microsporidian taxa and can include several sporulation sequences among multiple host generations and intermediate hosts (Becnel and Andreadis 1999, Keeling and Fast 2002). Some genera feature two sporulation sequences that produce morphologically distinct cells and target different tissues. The first or primary sporulation sequence yields auto-infective primary spores to spread within the same host, whereas the secondary sporulation sequence yields spores intended for excretion from the current host and transmission to new hosts (Keeling and Fast 2002, Becnel and Andreadis 1999, Goertz and Hoch 2008a). These environmental spores can be transmitted among compatible hosts within the same host generation (horizontal transmission) or between host generations (vertical transmission). Horizontal transmission can occur via the ingestion of food sources contaminated by infected feces or cadavers, via healthy host cannibalism of moribund individuals, and by host parasitoids with contaminated ovipositors (Becnel and Andreadis 1999). Vertical transmission from the maternal parent can occur by infection from ovaries to egg (transovarial) or by larval exposure to the infected surface of the egg during eclosion (Becnel and Andreadis). Paternal transmission is rare, but eggs may be infected during mating (Becnel and Andreadis 1999).

Under certain *in vitro* conditions, microsporidia can be grown, to some degree, in a variety of cell and tissues cultures, which might suggest low theoretical host specificity (Becnel and Andreadis 1999). *In vivo* studies on microsporidia host specificity in insects indicate infection among non-host genera within a given insect order is possible, while infection occurs across phyla (insect-to-mammal) is generally rare (Becnel and Andreadis 1999), more recent studies have reported *Anncaliia algerae* in several tissues of human patients (Solter *et al.* 2012). Under field conditions, Solter *et al.* 1997 found an *Endoreticulatus* sp. from Portugal capable of infecting many non-target insects and thus recommended the species not be considered as a candidate classical biological control method for North American gypsy moth (Becnel and Andreadis 1999).

Three genera of microsporidia have been found in European gypsy moth populations, though none so far have been identified in North American populations (McManus and Solter 2003). Microsporidia tend to persist at low levels in gypsy moth populations in periods between outbreaks, but can occasionally become a significant mortality factor in the gradation period preceding a gypsy moth outbreak (McManus and Solter 2003), leading to chronic disease causing delayed development, death, and increased vulnerability to parasitoid attack (McManus and Solter 2003), albeit at some detriment to the parasitoids (Hoch *et al.* 2000).

The three genera of microsporidan pathogens have differential life cycles and infection patterns in gypsy moth, which in turn affects their relative mortality and infectivity. Goertz and Hoch (2008a, 2008b) examined how these infection patterns manifest in horizontal and vertical transmission in gypsy moth. The midgut-infecting *Endoreticulatus schubergi* exhibited low virulence, but infected larvae produced copious amounts of spores in feces, indicative of its principle means of horizontal transmission. Transovum vertical transmission of *E.schubergi* spores is possible, but the resulting larvae showed low infection rates. *Vairimorpha disparis* infected and produced primary spores in the midgut, and environmental spores in fat bodies that caused high (90%) mortality and spore-laden cadavers that mediated horizontal transmission. Vertical transmission in *V. disparis* was negligible (Goertz and Hoch 2008a, Goertz and Hoch 2008b).

Nosema lymantriae infects and produces primary spores in the midgut and secondary (environmental) spores in other target tissues, including the silk glands, gonads, fat bodies, and malphigian tubules. *N. lymantriae*-infected larvae showed mortality and numbers of spores per cadaver and per fresh mass comparable to *V. disparis*. Total spores released in feces were lower than *E. schubergi*, but *N. lymantriae* released spores over a longer period, with greater continuity, and high rates of subsequent infectivity, depending on collection period (Goertz and Hoch 2008a). Unlike *E. schubergi,* which produced fecal spores from midgut infection, environmental spores of *N.lymantriae*-contaminated feces via the Malphigian tubules (Goertz and Hoch 2008a). Additionally, *N. lymantriae* spores infected 35-72% of hatched larvae by transovarial transmission (Goetz and Hoch 2008b). The diversity and efficacy of its transmission pathways therefore make *N. lymantriae* the most appropriate candidate for further investigations on its potential use as a biocontrol agent for gypsy moth.

Previous studies on the interactions between microsporida and other gypsy moth control methods have focused on parasitoids, chemical pesticides, and NPV. Hoch *et al.* (2000) found higher intensities of *Vairimorpha* infections in gypsy moth larvae parasitized by a braconid parasitoid, albeit at the cost of delayed development, reduced mass and longevity in parasitoid larvae. Additionally, Hoch and Schopf (2001) observed interactive effects in gypsy moth with different combinations of *Vairimorpha* infection and venom/PDV treatments. Combined treatments showed higher growth rates, delayed development, and increased infection intensity relative to microsporidia- and venom/PDV-only treatments, respectively (Hoch and Schopf 2001). Dimilin, a formerly prominent insecticide against gypsy moth in Europe, significantly reduces the number and subsequent infectivity of *N. lymantriae* spores, presumably due to its inhibition of chitin synthesis (Goertz *et al.* 2004). Bauer *et al.* (1998) compared simultaneous and sequential mixed infections of *Nosema* sp. and NPV in gypsy moth and found increased susceptibility to viral infection as well as reduced *Nosema* spore and viral polyhedra content in larvae when *Nosema* infection preceded NPV inoculation.

Published studies on the interactions between Bt and *Nosema* have so far been limited to *Nosema pyrausta* in the European corn borer (*Ostrinia nubilalis* Hubner), where a continuous, Bt-laced diet reduced spore number in larvae and frequently led to larval death before microsporidian transmission, therefore reducing the potential efficacy of *N. pyrausta* as a biocontrol agent in Bt-corn (Pierce *et al.* 2001). Mayrhofer (unpublished) examined the effects of

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lethal and sub-lethal concentrations of Bt on gypsy moth simultaneously inoculated with one hundred or one thousand *N. lymantriae* spores at the second or third instar, respectively. Increasing Bt concentration was found to inhibit feeding of the inoculated diet block. Mortality of Bt and *N. lymantriae* infected individuals was also dependent on Bt concentration, where Bt-mediated deaths predominated only at higher Bt concentrations (≥ 2.0 infection units (IU) of Bt). At lower Bt concentrations, *N. lymantriae* infections, rather than Bt, caused larval mortality. *N. lymantriae* spore counts in infected larvae also decreased significantly at lethal Bt concentration. This strongly suggests that in mixed-infected larvae, one or the other pathogens cause mortality. Therefore, above a lethal Bt concentration threshold, Bt acts faster to kill larvae before the *N. lymantriae* spores proliferate and the onset of systematic infection. At sub-lethal Bt concentrations, however, larvae survive long enough to allow for systemic and fatal *N. lymantriae* infections.

Mayrhofer (unpublished) did not address how Bt concentrations affect the other pathway of *N. lymantriae* horizontal transmission in gypsy moth: transmission via contaminated feces. Feces contamination is a consequence of environmental spore deposition from the Malpighian tubules, one of the target tissues of *N. lymantriae*. The following study examined the effects Bt concentration on *N. lymantriae* spore counts in feces, the viability of spores contained in feces, and the effects of Bt on vertical transmission as observed from egg masses produced from the Mayrhofer (unpublished) study.

2 Materials and Methods

2.1 Insects and pathogens

Lymantria dispar larvae (New Jersey Standard Strain) derived from egg masses from the USDA-APHIS Otis Development Center (Massachusetts, USA) were reared on a meridic wheat germ-based diet (Bell *et al.* 1981, diet changed every 2-3 days,) in climate chambers with a 16L:8D photoperiod at 24º C (day) and 18º C (night). A 12,700-infection units (IU) stock concentration of *Bacillus thuringiensis* (Bt) var. *kurstaki* (Biobit XL, Valent Biosciences Corp., Libertyville, Ill., USA) was used for Bt inoculations. Microsporidia inoculations utilized *Nosema lymantriae* (Isolate No. 1996-A) spores originally isolated from the silk glands of *Lymantria dispar* larvae collected near Levishte, Bulgaria (Laboratory of Dr L.F. Solter, Illinois Natural History Survey, Urbana-Champaign, IL, USA). Spore inoculum used for this study was stored in liquid nitrogen at the Laboratory of Dr. Gernot Hoch at the Institute of Forest Entomology, Forest Pathology and Forest Protection, University of Natural Resources and Life Sciences, Vienna, Austria.

2.2 Horizontal Transmission

2.2.1 Inoculation

Freshly molted, third-instar *L. dispar* larvae, starved for 24 hours, were placed individually in 24-well plates and fed a 1mm^3 diet block laced with 1 µl of one of the following treatment solutions (treatment name in parentheses): dH$_2$O (control); 0.5 IU of Bt (0.5Bt); 1 IU Bt (1Bt); 2 IU Bt (2Bt); 1 x 10^3 spores of *N. lymantriae* (NL); 0.5 IU Bt + 1 x 10^3 spores of *N. lymantriae* (NL+0.5Bt); 1 IU Bt + 1 x 10^3 spores of *N. lymantriae* (NL+1Bt); or 2 IU Bt + 1 x 10^3 spores of *N. lymantriae* (NL+2Bt). After 24 hours, only larvae that consumed the entire diet block were considered inoculated and were moved to individual petri dishes for subsequent use in the study.

2.2.2 Larval development and mortality

During the course of development, initial weight, weight at death or pupation, molting dates, and date of death were recorded. Death by microsporidiosis of larvae treated with *N. lymantriae* was verified by observing a cross-section of the second abdominal segment under phased-contrast microscopy at 400x. This procedure was conducted twice.

2.2.3 Number and infectivity of spores in feces

For each *N. lymantriae*-inoculated treatment group, feces were collected during three phases: from the beginning to the end of the fourth instar; the first five days of the fifth instar; and the second five days of the fifth instar. Fecal pellets were then frozen (-18° C) for storage. For extraction of spores, frozen fecal pellets were thawed, suspended and homogenized in 5 mL dH$_2$O, then filtered and centrifuged at 1300g for 15 min. (Goertz and Hoch 2008a). After discarding the supernatant, the resulting pellet was re-suspended in 1mL dH$_2$O and immediately counted using a Neubauer Improved hemocytometer under phase-contrast microscopy (400x). Spores were again frozen (-18° C) for storage until subsequent use.

To determine the viability and infectivity of spores collected from feces of the *N. lymantriae*-only and *N.lymantriae* + Bt treatments, spores of each treatment group were pooled into same-treatment groups and diluted to a standard concentration (1 x 10^3 spores/μl). These spore solutions were applied to diet blocks for inoculation of third-instar larvae as described above. Larvae that consumed the entire diet block were transferred into ventilated cups containing meridic diet and reared under the aforementioned conditions in climate chambers. At 20 days post infection (d.p.i.), cross-sections of the second abdominal segment were prepared and observed under phase-contrast microscopy (200x and 400x) to confirm presence/absence of *N. lymantriae* environmental spores. This experiment was performed twice.

2.3 Vertical Transmission

Egg masses resulting from mating parents with mixed Bt + *N. lymantriae* infections from a previous study (Table 4) were cut in half and placed either into ventilated rearing boxes containing meridic diet or into petri dishes lacking diet. For the non-starved egg mass halves, the number of hatched larvae was recorded. At 14 days post incubation, 30 larvae from each egg mass derived from a *N. lymantriae*-infected mother were dissected by a cross-section of the second abdominal segment and observed under phased-contrast microscopy (400x) for the presence/absence of *N. lymantriae* spores to estimate the proportion of infected larvae. For the starved egg mass halves, the number of hatched larvae were relocated daily and then moved to empty petri dishes were the number of deaths per days was recorded to determine differences in survivability among treatments.

2.4 Statistical Analysis

The statistical software SPSS 18.0.0 (IBM, Armonk, NY, USA) was used to analyze data. Data sets were tested for normality using the Kolmogorov-Smirnov test. Normally-distributed data were analyzed using one-way ANOVA (Appendix) followed by pairwise comparison by Bonferroni post-hoc tests. Non-normal data were analyzed using the Krusak-Wallis *H*-test followed by Tukey-Kramer test for multiple pairwise comparisons. Frequency data was analyzed by χ^2 analysis.

3 Results

3.1 Larval Development

Development and weight data for the Bt-only and control treatments were separated by sex based on identification at adult eclosion or pupal weight. Among Bt treatments in the first trial, female 2Bt larvae had significantly longer third instars (dpi until molt to the fourth instar) than other Bt and control treatments, while male 2Bt larvae had significantly longer third instars than control and 0.5Bt larvae (Tables 1a and 2a). Female larvae from the 0.5Bt and 1Bt treatments exhibited significantly shorter fourth instars (dpi until molt to the fifth instar) than control and 2Bt larvae, but no significant differences in fourth instar length were observed in male larvae among Bt treatments. Differences in weight at 1 dpi, pupal weight, and pupation and adult eclosion dates were not significant among Bt treatments for female and male larvae (Tables 1a and 2a, Appendix). In the second trial, Bt-treated female larvae showed significantly longer third instars than controls but not among each other (Tables 1b and 2b, Appendix). Female fourth instar length was not affected. Male 2Bt larvae exhibited significantly longer third and fourth instars. Mean eclosion date for female 1Bt pupae was significantly longer than other treatments, but all other developmental data in Bt and control treatments were not significantly different in both sexes in the second trial (Appendix).

High mortality of *N. lymantriae*-treated larvae prior to pupation prevented the identification and separation of larvae by sex. Comparisons of developmental data between Bt and NL treatments were therefore not possible. In the first trial, larvae of the *N. lymantriae*–only (NL) treatment reached the fourth instar significantly sooner (5.5 dpi) than larvae with mixed infections (7.1-7.3 dpi), while no significant differences were observed in dpi to the fifth instar or in mean dpi at death (Tables 1a and 2a, Appendix). In the second trial, NL+2 Bt larvae had significantly longer third and fourth instars and delayed death, while NL+1 larvae exhibited longer third and fourth instars than NL and NL+0.5 Bt larvae (Tables 1b and 2b, Appendix). Differences in weight at 1 dpi and at death were not significant among *N. lymantriae* treatments (with or without Bt) in both trials (Appendix).

Table 1a First trial weights of larvae at different life stages for all treatments. Number of individuals indicated in parentheses. Statistical comparisons (One-way ANOVA, Bonferroni post-hoc. See Appendix) were made only within sex and within Bt-only or within *N. lymantriae* treatment sets; superscript letters indicate significant differences at α = 0.05.

Treatment	Weight 1 dpi [g] female/male	Weight at Death [g]	Weight of Pupae [g] female/male
Control (15)	0.029 ± 0.001^A (7) 0.029 ± 0.002^a (8)	n/a	1.83 ± 0.12 (7)^A 0.55 ± 0.04 (7)^a
0.5 Bt (15)	0.029 ± 0.001^A (6) 0.029 ± 0.002^a (9)	n/a	1.75 ± 0.33 (6)^A 0.59 ± 0.10 (9)^a
1.0 Bt (15)	0.029 ± 0.002^A (8) 0.027 ± 0.003^a (7)	n/a	2.01 ± 0.18 (8)^A 0.48 ± 0.03 (7)^a
2.0 Bt (15)	0.034 ± 0.002^A (8) 0.032 ± 0.002^a (7)	n/a	1.93 ± 0.13 (8)^A 0.48 ± 0.04 (7)^a
NL Only (15)	0.025 ± 0.001^a (15)	0.30 ± 0.02^a (15)	n/a
NL + 0.5 Bt (15)	0.027 ± 0.001^a (15)	0.27 ± 0.03^a (15)	n/a
NL + 1 Bt (15)	0.026 ± 0.001^a (15)	0.25 ± 0.04^a (15)	n/a
NL + 2 Bt (15)	0.028 ± 0.001^a (15)	0.38 ± 0.09^a (15)	n/a

Table 1b Second trial weights of larvae at different life stages for all treatments. Number of individuals indicated in parentheses. Statistical comparisons (One-way ANOVA, Bonferroni post-hoc. See Appendix) were made only within sex and within Bt-only or within *N. lymantriae* /mixed treatment sets; superscript letters indicate significant differences at α = 0.05.

Treatment	Weight 1 dpi [g] female/male	Weight at Death [g]	Weight of Pupae [g] female/male
Control (10)	0.023 ± 0.001^A (4) 0.021 ± 0.002^a (6)	n/a	2.02 ± 0.07^A (4) 0.63 ± 0.03^a (6)
0.5 Bt (10)	0.028 ± 0.003^A (6) 0.022 ± 0.003^a (4)	n/a	1.92 ± 0.20^A (6) 0.58 ± 0.06^a (4)
1.0 Bt (10)	0.026 ± 0.002^A (3) 0.022 ± 0.003^a (7)	n/a	2.41 ± 0.23^A (3) 0.58 ± 0.02^a (7)
2.0 Bt (6)	0.025 ± 0.001^A (4) 0.020 ± 0.003^a (2)	n/a	2.10 ± 0.56^A (4) 0.60 ± 0.03^a (2)
NL Only (25)	0.022 ± 0.001^a (25)	0.61 ± 0.10^a (18)	2.10 (1) 0.62 (1)
NL +0.5 Bt (25)	0.023 ± 0.001^a (25)	0.77 ± 0.11^a (22)	2.01 (1) 0.16 (1)
NL +1 Bt (25)	0.023 ± 0.001^a (25)	0.51 ± 0.07^a (17)	1.89 (1) 0.54 (1)
NL +2 Bt (25)	0.027 ± 0.001^a (25)	0.81 ± 0.14^a (17)	2.24 (1) 0.64 (1)

Table 2a Days post-infection (dpi) of different developmental phases, first trial. Statistical comparisons (One-way ANOVA, Bonferroni post-hoc. See Appendix) were made only within sex and within Bt-only or within *N. lymantriae* /mixed treatment sets; superscript letters indicate significant differences at α = 0.05.

Treatment	4th Instar [dpi] female/male	5th Instar [dpi] female/male	Pupation [dpi] female/male	Adult [dpi] female/male	Death [dpi]
Control (15)	5.9 ± 0.1^A (7)	15.9 ± 0.3^B (7)	33.0 ± 1.9^A (7)	45.7 ± 1.0^A (7)	n/a
	5.4 ± 0.2^a (8)	12.3 ± 0.9^a (8)	24.8 ± 1.4^a (7)	43.1 ± 1.0^a (7)	
0.5 Bt (15)	6.0 ± 0.2^A (6)	15.3 ± 0.4^A (6)	32.4 ± 1.6^A (6)	47.0 ± 1.9^A (6)	n/a
	5.6 ± 0.8^a (9)	15.6 ± 0.8^a (9)	25.6 ± 1.1^a (9)	43.5 ± 0.9^a (9)	
1.0 Bt (15)	5.5 ± 0.7^A (8)	15.6 ± 0.5^A (8)	30.2 ± 1.1^A (8)	49.8 ± 3.8^A (8)	n/a
	6.2 ± 0.5^{ab} (7)	15.7 ± 0.7^a (7)	27.0 ± 1.3^a (7)	44.0 ± 1.6^a (6)	
2.0 Bt (15)	8.1 ± 0.4^B (8)	17.6 ± 0.6^B (8)	33.1 ± 1.0^A (8)	45.0 ± 0.7^A (7)	n/a
	8.0 ± 0.7^b (7)	16.3 ± 0.9^a (7)	28.7 ± 1.9^a (7)	45.5 ± 0.6^a (5)	
NL Only (15)	5.5 ± 0.1^a (15)	15.7 ± 0.3^a (6)	n/a	n/a	21.1 ± 1.0^a (15)
NL + 0.5 Bt (15)	7.3 ± 0.3^b (15)	18.0^a (1)	n/a	n/a	19.5 ± 0.8^a (15)
NL + 1 Bt (15)	7.1 ± 0.3^b (15)	15.5 ± 1.5^a (2)	n/a	n/a	18.8 ± 0.9^a (15)
NL + 2 Bt (15)	7.1 ± 0.4^b (15)	15.3 ± 0.2^a (7)	n/a	n/a	20.8 ± 0.6^a (15)

Table 2b Days post-infection (dpi) of different developmental phases, second trial. Statistical comparisons (One-way ANOVA, Bonferroni post-hoc. See Appendix) were made only within sex and within Bt-only or within *N. lymantriae*/mixed treatment sets; superscript letters indicate significant differences at α = 0.05.

Treatment	4th Instar [dpi] female/male	5th Instar [dpi] female/male	Pupation [dpi] female/male	Adult [dpi] female/male	Death [dpi]
Control (10)	6.8 ± 0.3^A (4)	13.3 ± 0.3^A (4)	26.5 ± 0.6^A (4)	42.3 ± 0.7^A (3)	n/a
	6.3 ± 0.2^a (6)	12.7 ± 0.5^a (6)	25.7 ± 0.5^a (6)	44.5 ± 0.9^a (4)	
0.5 Bt (10)	7.2 ± 0.2^B (6)	13.5 ± 0.6^A (6)	29.5 ± 1.0^A (6)	45.2 ± 1.1^A (5)	n/a
	6.8 ± 0.8^a (4)	13.3 ± 0.9^a (4)	28.0 ± 1.6^a (4)	46.0 ± 1.2^a (4)	
1.0 Bt (10)	8.0 ± 0.6^B (3)	15.0 ± 0.6^A (3)	31.7 ± 2.3^A (3)	49.5 ± 1.5^B (2)	n/a
	6.7 ± 0.3^a (7)	13.0 ± 0.4^a (7)	25.4 ± 0.5^a (7)	45.1 ± 0.5^a (7)	
2.0 Bt (6)	8.6 ± 0.4^B (4)	15.2 ± 0.7^A (4)	29.2 ± 0.5^A (4)	48.0 ± 0.0^A (3)	n/a
	8.5 ± 0.5^b (2)	16.0 ± 2.0^b (2)	29.0 ± 0.5^a (2)	48.0 ± 0.0^a (1)	
NL Only (25)	6.0 ± 0.1^a (25)	12.1 ± 0.2^a (21)	31 (1)	n/a	19.5 ± 0.8^a (25)
			24 (1)	43 (1)	
NL + 0.5 Bt (25)	5.8 ± 0.2^a (25)	12.0 ± 0.2^a (20)	26 (1)	43 (1)	19.9 ± 0.9^a (25)
			24 (1)	44 (1)	
NL + 1 Bt (25)	7.2 ± 0.2^b (25)	13.3 ± 0.3^b (25)	33 (1)	48 (1)	20.2 ± 0.7^a (25)
			26 (1)	n/a	
NL + 2 Bt (25)	7.8 ± 0.2^b (25)	14.4 ± 0.2^c (22)	29 (1)	n/a	24.3 ± 1.5^b (25)
			28 (1)	46 (1)	

3.2 Cumulative and Stage Specific Mortality

For both trials, none of the larvae infected only with Bt died during the third, fourth or fifth instars, though some pupae in control and Bt treatments failed to ecolse to adult moths (Figs. 1a and 1b). In first trial, 40% of larvae in the NL-only treatment survived to the fifth instar (Fig. 1a), which occurred at 15-16 dpi. Mortality increased from 46% at 21 dpi to 73% at 22 dpi (Fig. 2a), with median death at 21 dpi. Ninety-three percent of NL-only larvae were dead by 28 dpi, and 100% by 29 dpi. For NL+0.5Bt larvae, one individual (6.67%) survived to the fifth instar at 18 dpi. Fifty percent of larvae were dead by 19 dpi, 73% by 20 dpi, 93% at 24 dpi, and all were dead by

27 dpi (Fig. 2a). Thirteen percent (two individuals) of NL+1Bt larvae survived to the fifth instar (15-16 dpi). Mortality of this treatment increased from 26% to 93% from 17 to 20 dpi (median death 18 dpi), but 100% mortality did not occur until 30 dpi (Fig. 2a). Forty-seven percent of NL+2Bt larvae survived to the fifth instar (15-16 dpi). Only 50% of larvae were dead by 21 dpi, but mortality increased to 86% by 23 dpi and 100% at 24 dpi (Table 2a, Figs. 1a and 2a). Median dpi at death was significantly different among treatment groups (χ^2 = 9.33, d.f. =3, p = 0.025). None of the larvae treated with *N. lymantriae* survived past the fifth instar, but all *N-lymantriae*-inoculated larvae exhibited extensive microsporidia infection when observed by dissection.
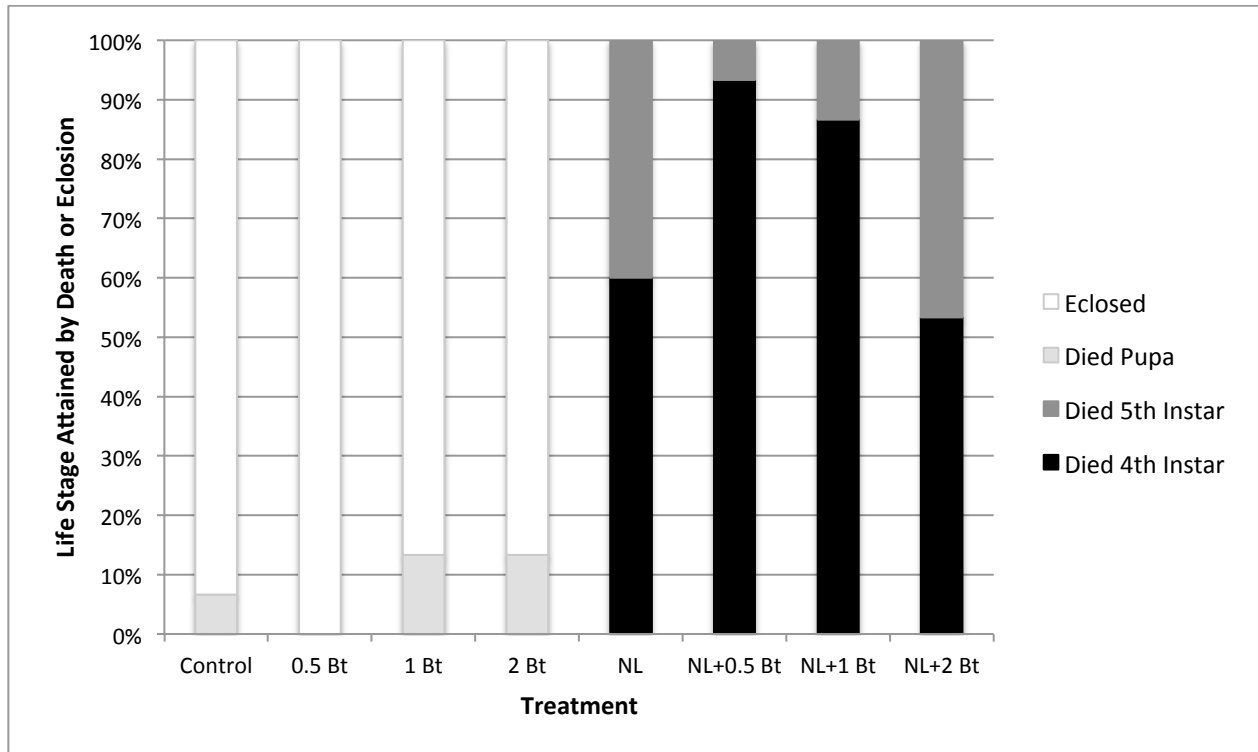
Fig. 1a Mortality of larvae at different developmental phases, first trial.
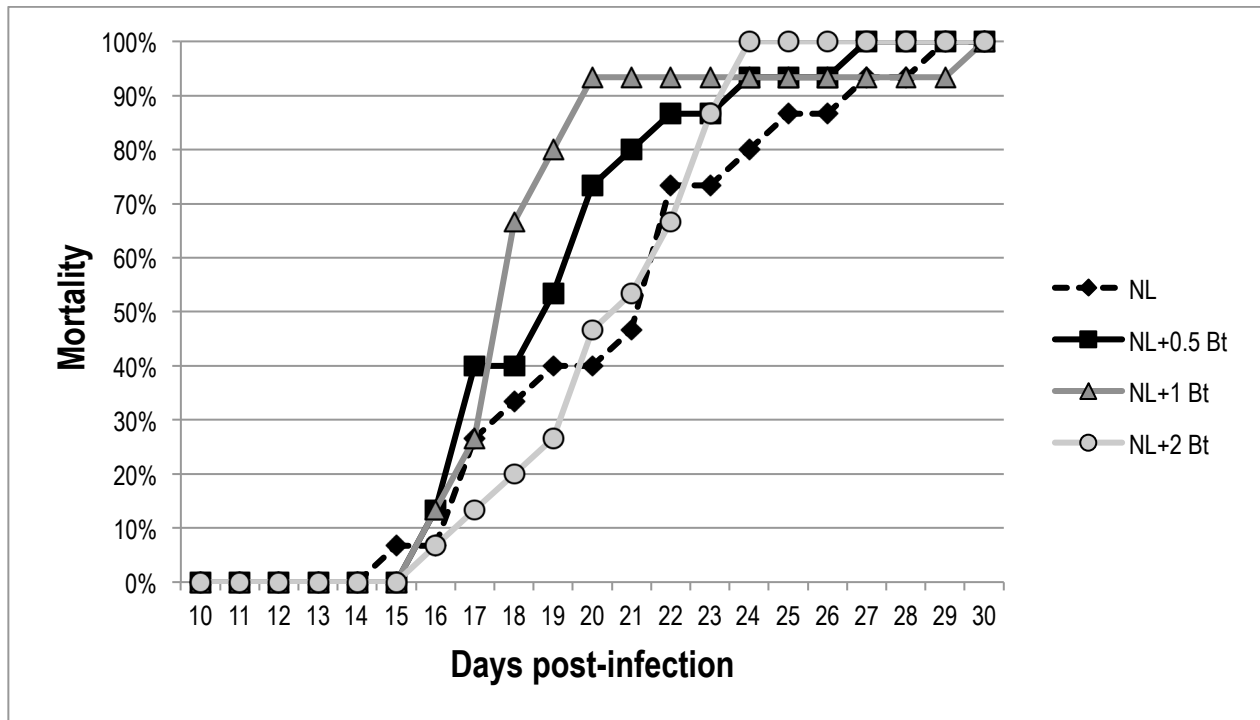
Fig. 2a Mortality curves of mixed infection treatments, first trial.

In the second trial, 84% of NL-only larvae survived to the fifth instar (Fig 1b, 12 dpi). Half were dead by 19 dpi, 73% by 20 dpi, 95% by 25 dpi and total mortality was reached at 27 dpi. Eighty-seven percent of NL+0.5Bt larvae survived to the fifth instar (Fig. 1b, 12 dpi). Sixty-four percent of larvae in this treatment group were dead at 19 dpi, 95% at 27 dpi, and 100% at 29 dpi (Fig. 2b). All NL+1Bt larvae survived to the fifth instar (13 dpi), but 50%, 96% and 100% of larvae had died by 20 dpi, 26 dpi, and 28 dpi, respectively (Fig. 2b). For larvae treated with NL+ 2Bt, 88% survived to the fifth instar (14 dpi). Mortality of larvae in this treatment group increased from 47% at 22 dpi to 63% at 23 dpi, but did not achieve 89% mortality until 31 dpi and 100% dpi at 32 dpi (Fig. 2b). Only 8-9% (2 larvae) in each of the four treatment groups survived to pupation. Only 1 adult emerged from each group, except NL+0.5Bt, where both pupae eclosed to adult moths (Table 2b, Figs 1b and 2b). Median dpi at death was significantly different among treatment groups (χ^2 =7.87, d.f. =3, p = 0.049). All *N. lymantriae*-inoculated larvae or pupa that did not survive to adulthood were confirmed to have died from microsporidiosis.
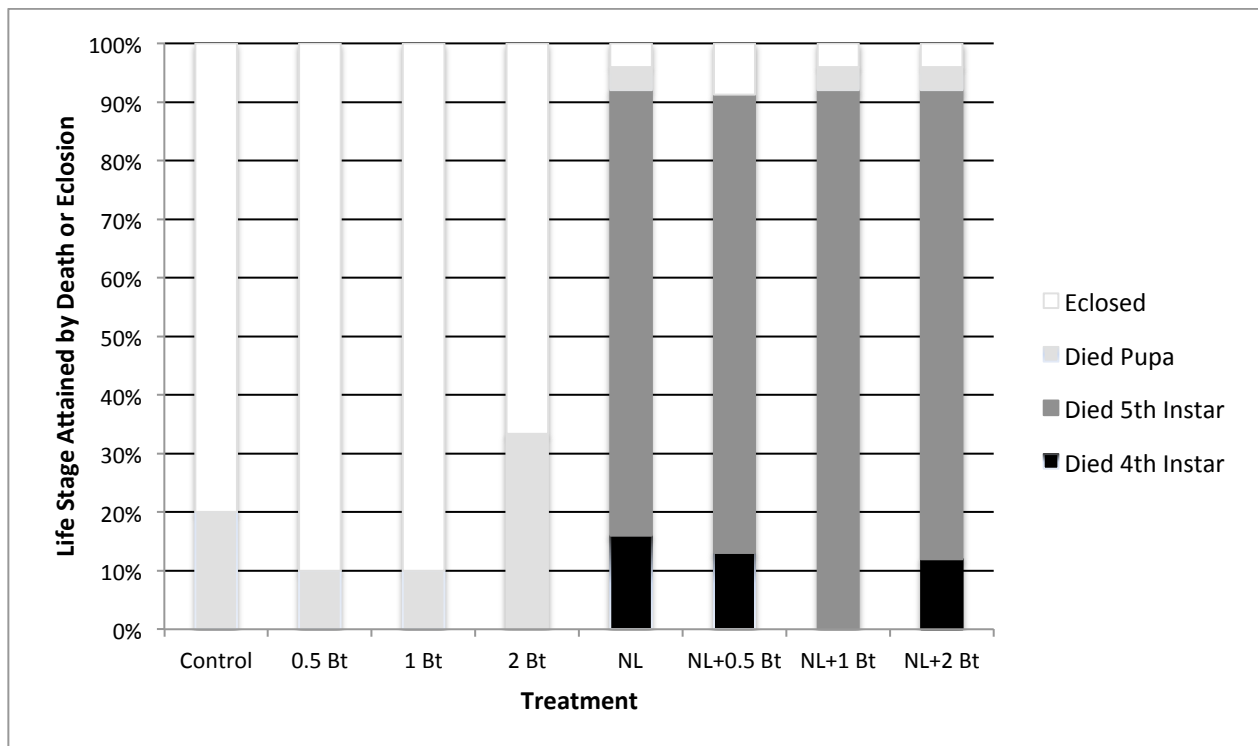
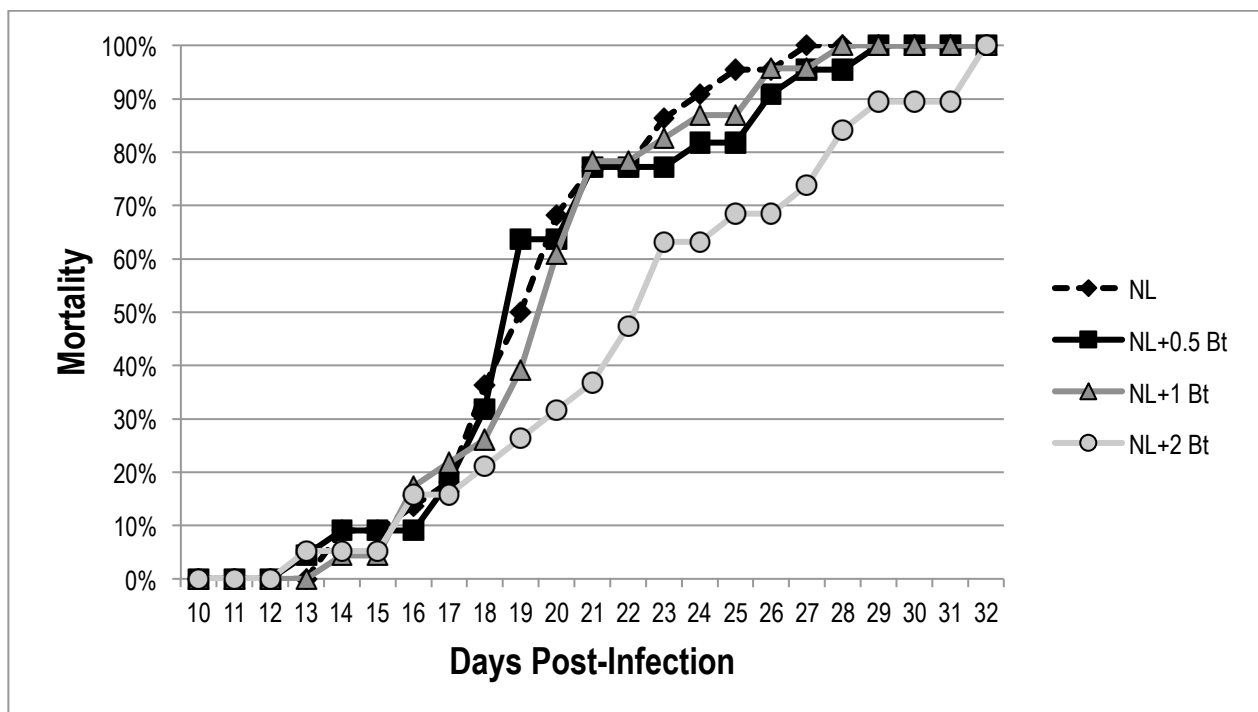**Fig. 1b** Mortality of larvae at different developmental phases, second trial.

Fig. 2b Mortality curves of mixed infection treatments, second trial.

3.3 Amount of Spores in Feces

Spore number exhibited similar fluctuations in ranges within and among feces collection periods in the first trial, but mean spore number was not significantly different (Kruskal-Wallis H /Tukey-Kramer, $p > 0.05$) among treatments within collection phases (Table 3a and Figs. 3a-c). Median spore number in feces collected in the fourth instar ranged from 1.2×10^6 (NL) to 1.9×10^6 (NL+0.5Bt) spores; minimum and maximum values from 1.2×10^5 (NL) to 6.0×10^5 (NL+1 Bt) and 1.7×10^7 (NL+1 Bt) to 3.1×10^7 spores, respectively (Fig. 3a). Among feces collected during the first five days of the fifth instar (Fig. 3b), two treatments had feces from only one individual each: NL+0.5 Bt with 2.1×10^7 spores and NL+1 Bt with 6.8×10^5 spores. The NL-only larvae produced feces with spore counts ranging from 4.8×10^6 to 2.0×10^7 and a median of 1.3×10^7 spores, whereas NL+2 Bt spore counts varied from 4.8×10^6 to 2.9×10^7 spores and a median of 1.3×10^7 spores. No larvae from the NL+0.5Bt treatment survived to produce feces during the second five days of the fifth instar (Fig. 3c). However, feces from the single NL+1Bt larvae increased to 3.7×10^6 spores during this collection phase. For the remaining treatments, spore counts ranged from 5.5×10^6 to 2.2×10^7 spores (median: 1.4×10^7 spores) in NL-only feces, and 2.8×10^6 to 2.5×10^7 spores' (median: 1.8×10^7 spores) for feces collected from NL+2Bt treated larvae (Fig. 3c).

Table 3a Mean spore number in feces among treatments and collection phase, first trial. Statistical comparisons (Kruskal-Wallis H-test (H-values: fourth instar, 4.654; fifth instar days 1-5, 4.654; fifth instar days 6-10, 0.810) followed by Tukey-Kramer pairwise comparisons, $p < 0.05$) were made only among treatments within a given collection phase. The same letters in each row indicated no statistical difference among treatments. Data with standard error of zero indicates feces were collected from only one individual.

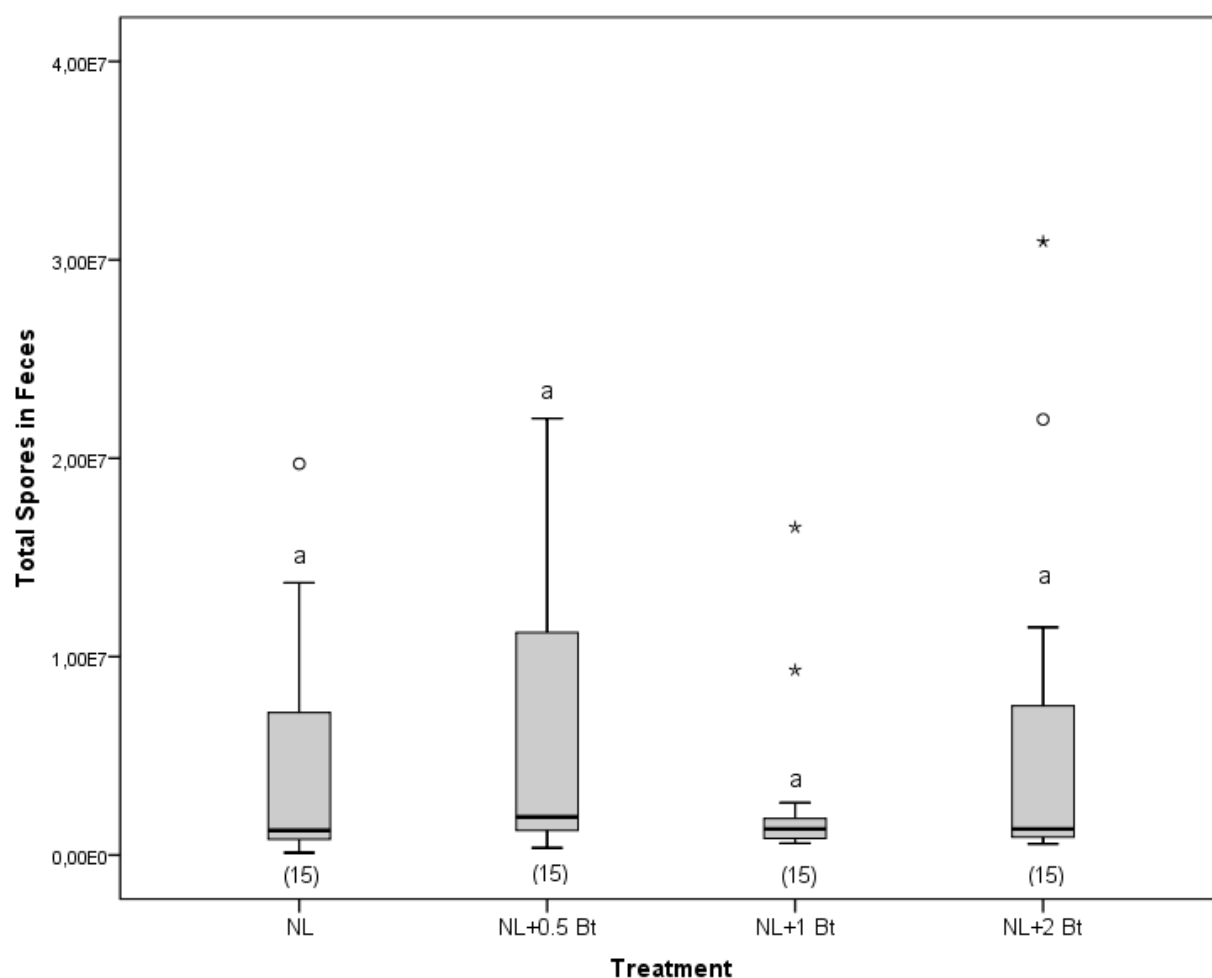| Mean Spore No.	NL Only	NL+0.5 Bt	NL+1 Bt	NL+2 Bt
Fourth Instar	$4.8 \times 10^6 \pm 1.6 \times 10^6$ a	$6.3 \times 10^6 \pm 1.8 \times 10^6$ a	$2.8 \times 10^6 \pm 1.1 \times 10^6$ a	$6.0 \times 10^6 \pm 2.4 \times 10^6$ a
5th Instar, Days 1-5	$1.4 \times 10^7 \pm 2.7 \times 10^6$ b	$2.1 \times 10^7 \pm 0$ b	$6.8 \times 10^5 \pm 0$ b	$1.3 \times 10^7 \pm 2.9 \times 10^6$ b
5th Instar, Days 6-10	$1.4 \times 10^7 \pm 8.5 \times 10^6$ c	n/a	$3.7 \times 10^6 \pm 0$ c	$1.5 \times 10^7 \pm 6.6 \times 10^6$ c

Fig. 3a Ranges of number of spores extracted from fourth-instar feces, first trial. Number of samples in each treatment indicated in parentheses below boxplots. The same letter above each boxplot indicates no significant differences among means (see Table 3a). Boxplots represent minimum, first quartile, median, third quartile, and maximum total spore counts.
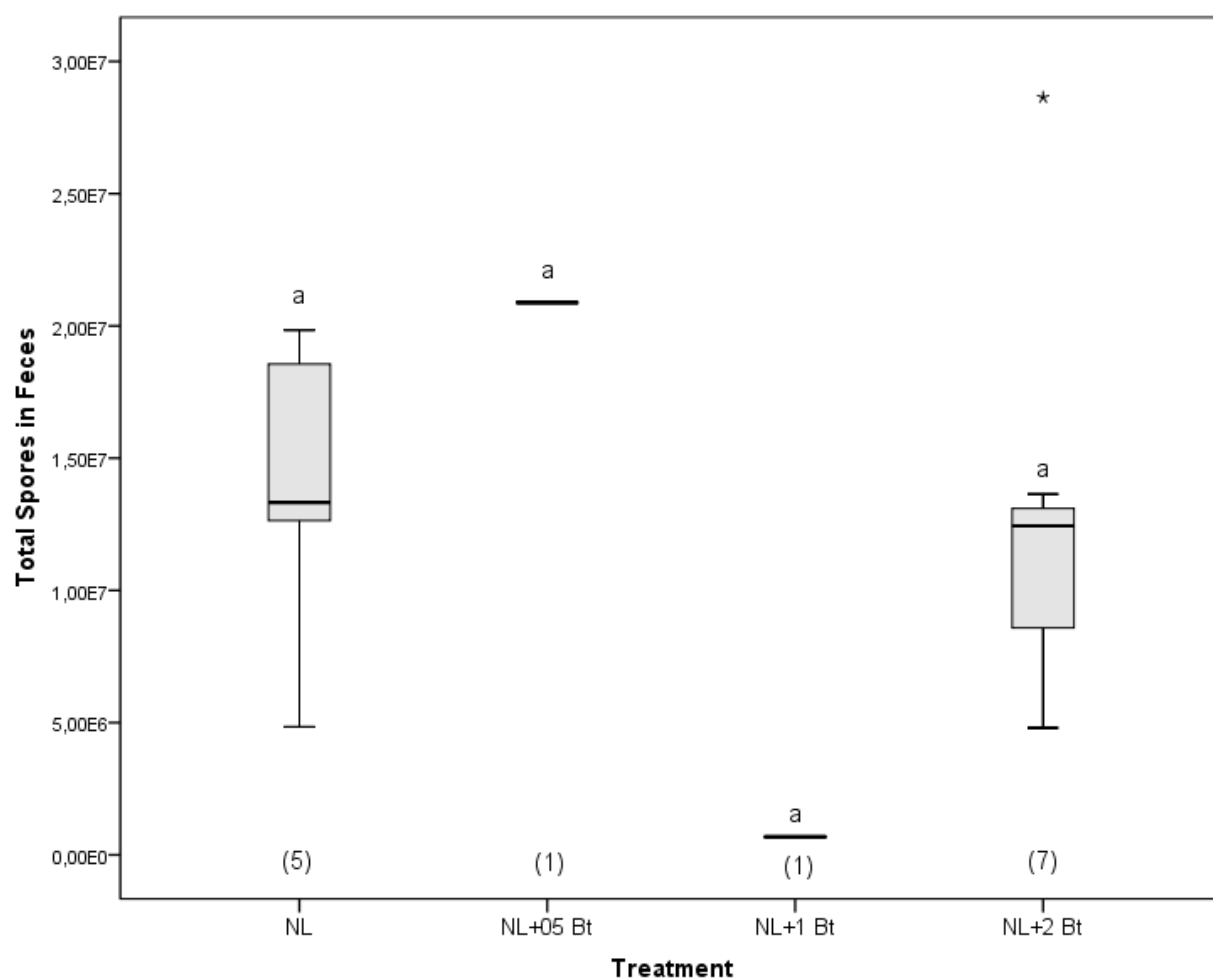
Fig. 3b Ranges of number of spores extracted from fifth-instar (days 1-5) feces, first trial. Number of samples in each treatment indicated in parentheses below boxplots. The same letter above each boxplot indicates no significant differences among means (see Table 3a). Boxplots represent minimum, first quartile, median, third quartile, and maximum total spore counts.
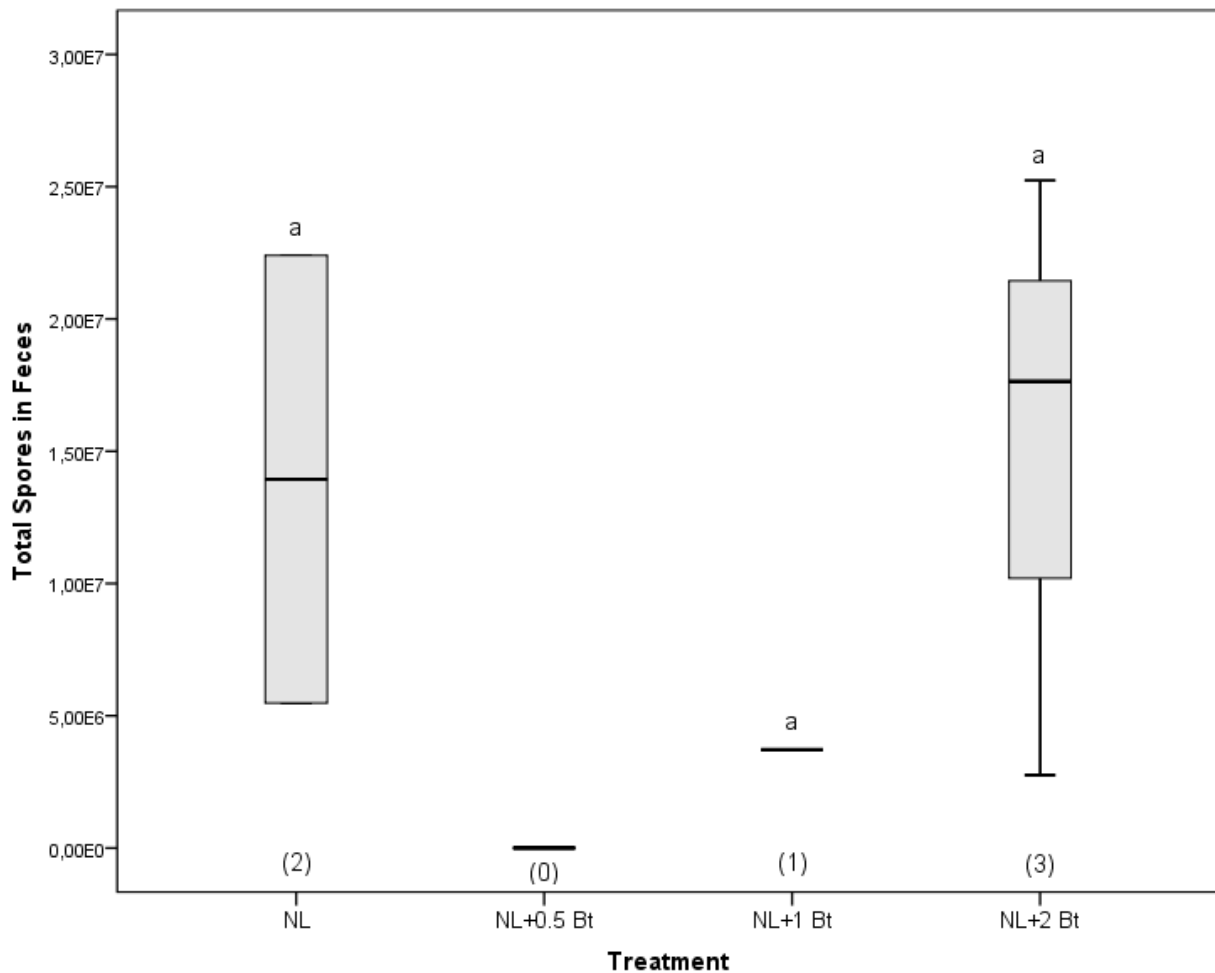
Fig. 3c Ranges of number of spores extracted from fifth-instar (days 6-10) feces, first trial. Number of samples in each treatment indicated in parentheses below boxplots. The same letter above each boxplot indicates no significant differences among means (see Table 3a). Boxplots represent minimum, first quartile, median, third quartile, and maximum total spore counts.

In the second trial, spore counts varied less among treatments and varied more among collection phases, but mean spore counts were again not significantly different (Kruskal-Wallis H /Tukey-Kramer, $p > 0.05$) among treatments within each collection phase (Table 3b and Figs. 4a-c). Feces collected during the fourth instar (Fig. 4a) had minimum spore counts ranging from 4.0 x10^4 spores in the NL-only treatment to 8.0 x10^4 spores in the other treatments, while maximum spore concentrations ranged from 1.2x10^6 (NL+0.5Bt) to 1.6x10^6 (NL+2 Bt) spores. Median values varied from 3.6x10^5 to 4.8x10^5 spores in NL+2 Bt and NL+1 Bt treatments, respectively. Minimum and maximum spore concentrations from feces collected during the first five days of the fifth instar (Fig. 4b) varied from 3.2 x10^5 (NL+ 2Bt) to 6.4 x10^5 (NL) spores and 8.1x10^6 (NL+0.5Bt) to 1.9x10^7 (NL+2 Bt) spores, respectively. Median spore concentrations from this collection phase ranged from 1.6x10^6 (NL) to 3.6x10^6 (NL+2 Bt) spores. All spore count values were higher in feces collected during the second five days of the fifth instar (Fig. 4c). Minimum values ranged from 2.8x10^5 (NL+2 Bt) to 5.4x10^5 (NL+1 Bt) spores; maximum values

from 1.2×10^7 (NL) to 4.2×10^7 (NL+1 Bt) spores, and medians from 7.3×10^6 (NL+0.5 Bt) to 1.2×10^7 (NL+1 Bt) spores.

Table 3b Mean spore number in feces among treatments and collection phase, second trial. Statistical comparisons (Kruskal-Wallis *H*-test (*H*-values: fourth instar, 0.399; fifth instar days 1-5, 3.059; fifth instar days 6-10, 1.151), followed by Tukey-Kramer pairwise comparisons, *p*< 0.05) were made only among treatments within a given collection phase. The same letters in each row indicated no statistical difference among treatments.

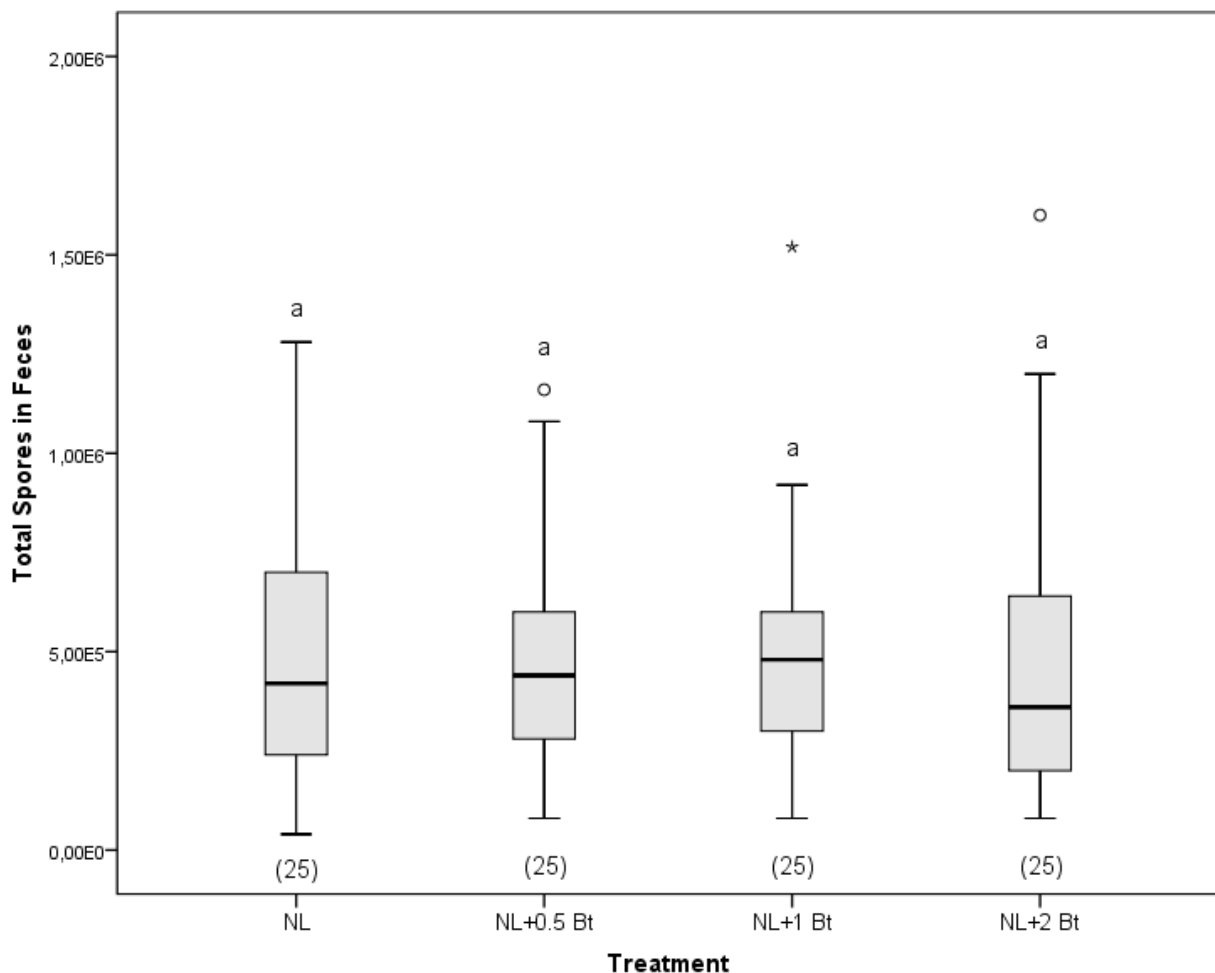| Mean Spore No.	NL Only	NL+0.5 Bt	NL+1 Bt	NL+2 Bt
Fourth Instar	$5.7 \times 10^5 \pm 6.5 \times 10^4$ a	$4.8 \times 10^5 \pm 5.5 \times 10^4$ a	$1.6 \times 10^5 \pm 6.5 \times 10^4$ a	$4.9 \times 10^5 \pm 7.8 \times 10^4$ a
5th Instar, Days 1-5	$3.8 \times 10^6 \pm 6.2 \times 10^5$ b	$2.9 \times 10^6 \pm 6.1 \times 10^5$ b	$6.4 \times 10^6 \pm 1.7 \times 10^6$ b	$5.7 \times 10^6 \pm 1.41 \times 10^6$ b
5th Instar, Days 6-10	$5.7 \times 10^6 \pm 1.2 \times 10^6$ c	$1.1 \times 10^7 \pm 3.8 \times 10^6$ c	$1.4 \times 10^7 \pm 4.4 \times 10^6$ c	$8.9 \times 10^6 \pm 2.2 \times 10^6$ c

Fig. 4a Ranges of number of spores extracted from fourth-instar feces, second trial. Number of samples in each treatment indicated in parentheses below boxplots. The same letter above each boxplot indicates no significant differences among means (see Table 3b). Boxplots represent minimum, first quartile, median, third quartile, and maximum total spore counts.
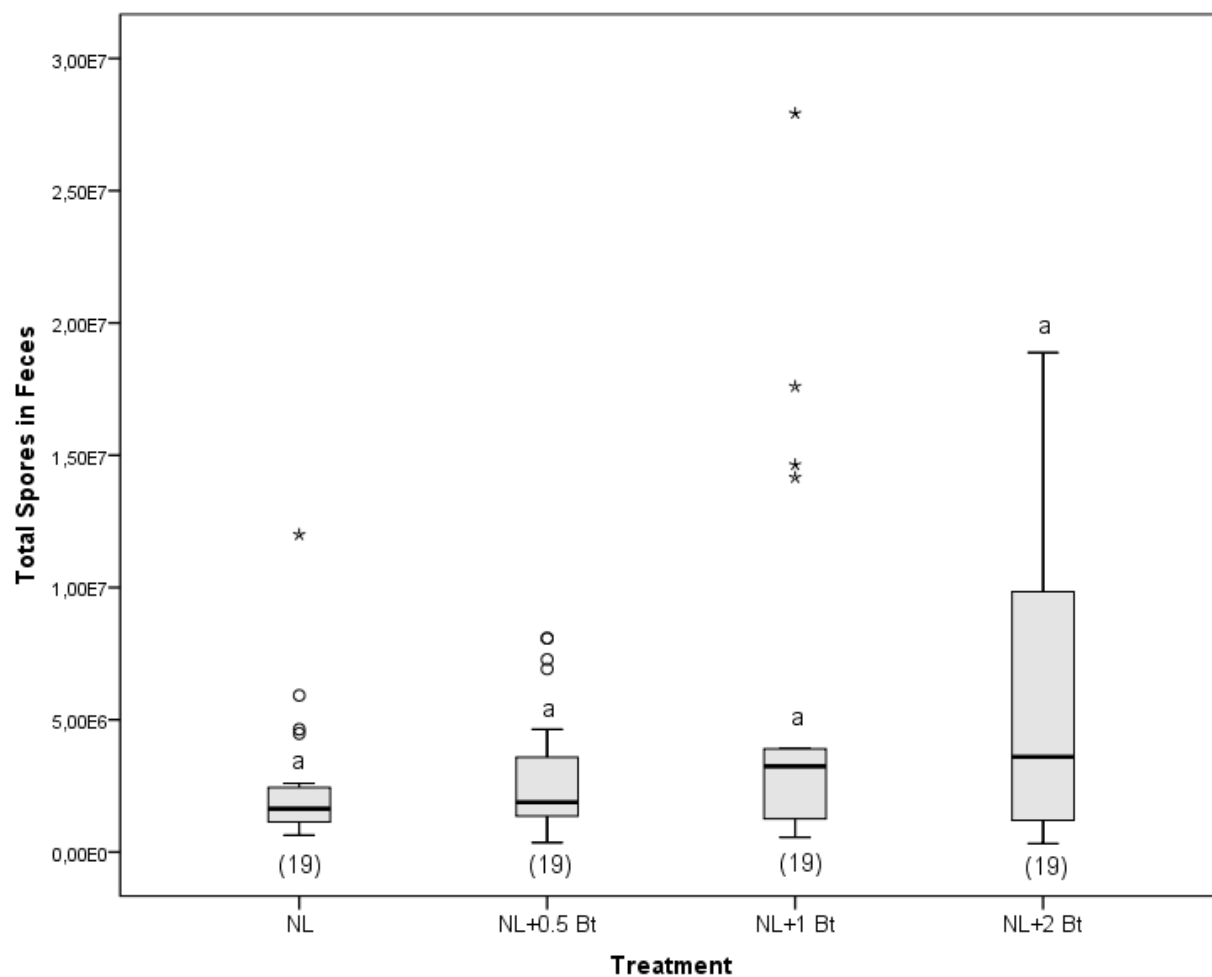
Fig. 4b Ranges of number of spores extracted from fifth-instar (days 1-5) feces, second trial. Number of samples in each treatment indicated in parentheses below boxplots. The same letter above each boxplot indicates no significant differences among means (see Table 3b). Boxplots represent minimum, first quartile, median, third quartile, and maximum total spore counts.
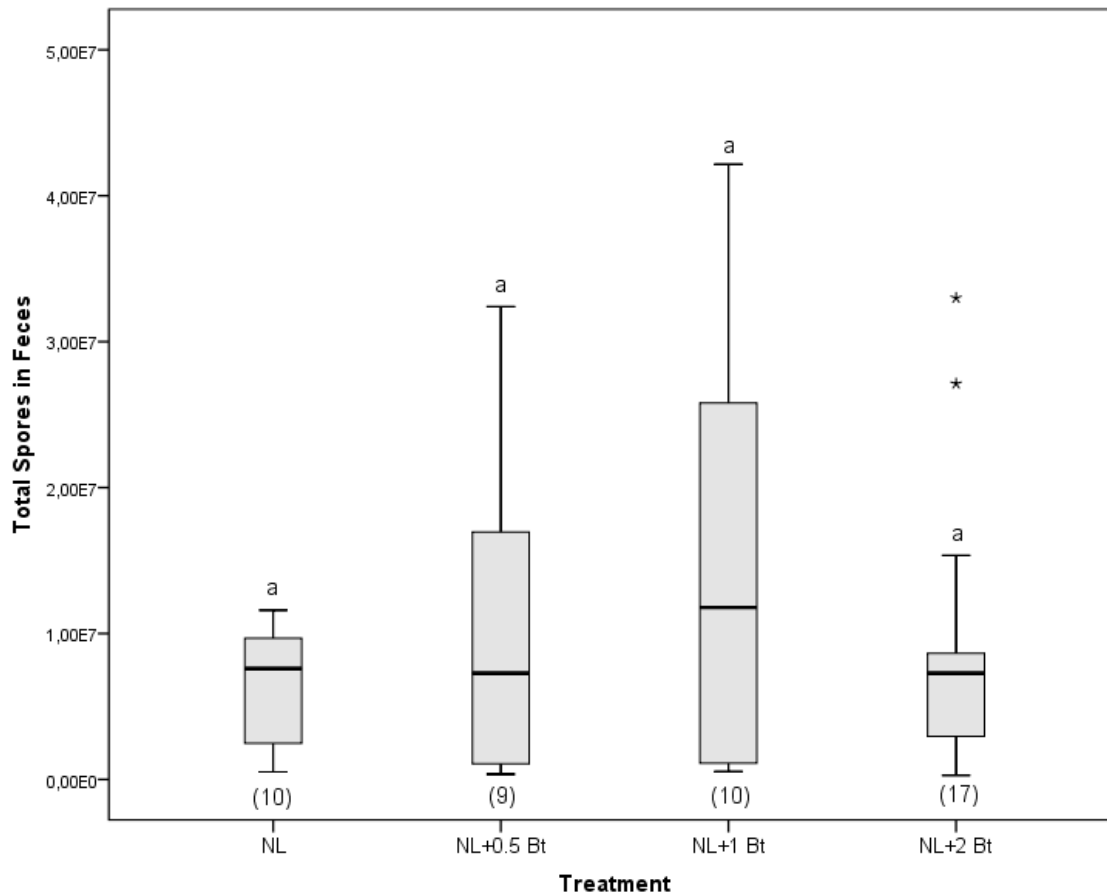
Fig. 4c Ranges of number of spores extracted from fifth-instar (days 6-10) feces, second trial. Number of samples in each treatment indicated in parentheses below boxplots. The same letter above each boxplot indicates no significant differences among means (see Table 3b). Boxplots represent minimum, first quartile, median, third quartile, and maximum total spore counts.

3.4 Infectivity of Spores Collected from Feces

Overall rates of infection were low for both trials (Figs. 5a and 5b). No significant differences (χ^2, $p > 0.05$) in infection rates among treatments were observed in any of the collection phases in both trials except L5 1-5 in the first trial, where spores derived from NL feces were more infective (35%) than NL+0.5 Bt (10%) and NL+1Bt and NL+ 2 Bt ($\chi^2 = 3.906$, $p = 0,0481$, Fig. 5a). Spores derived from second trial L5 1-5 feces had even lower infection rates (Fig. 5b), with NL and NL+2 Bt at 8% and NL+0.5Bt and NL+1Bt at 3%. For spores derived from fourth-instar feces, infection rates did not exceed 10-12% in two of the treatments in the first trial, while the remainder of treatments in both trials infection rates less than 2%. First trial L5 6-10 rates were 5% for both NL and NL+2Bt and 0% for NL+1Bt; no larvae from NL+0.5Bt survived to produce spores in feces and therefore were not included in the infectivity study. No larvae inoculated with NL spores became infected in the second trial L5 6-10, while the other treatments had 15% infection or less.
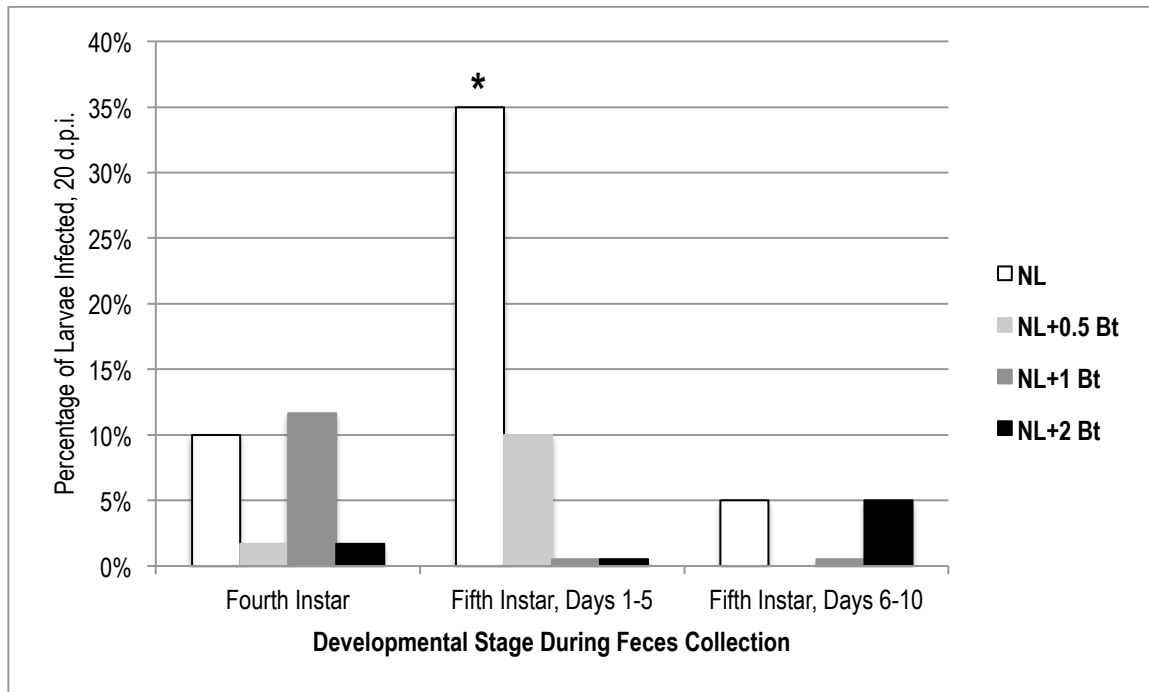
Fig. 5a Percentage of larvae successfully infected with spores derived from feces collected from larvae at different periods and of different mixed infection treatments, first trial. Star above bar indicates statistically significant difference in infectivity (χ^2 =3.906, d.f.=1, p = 0.0481); all other differences among treatments within each collection phase were not statistically significant.
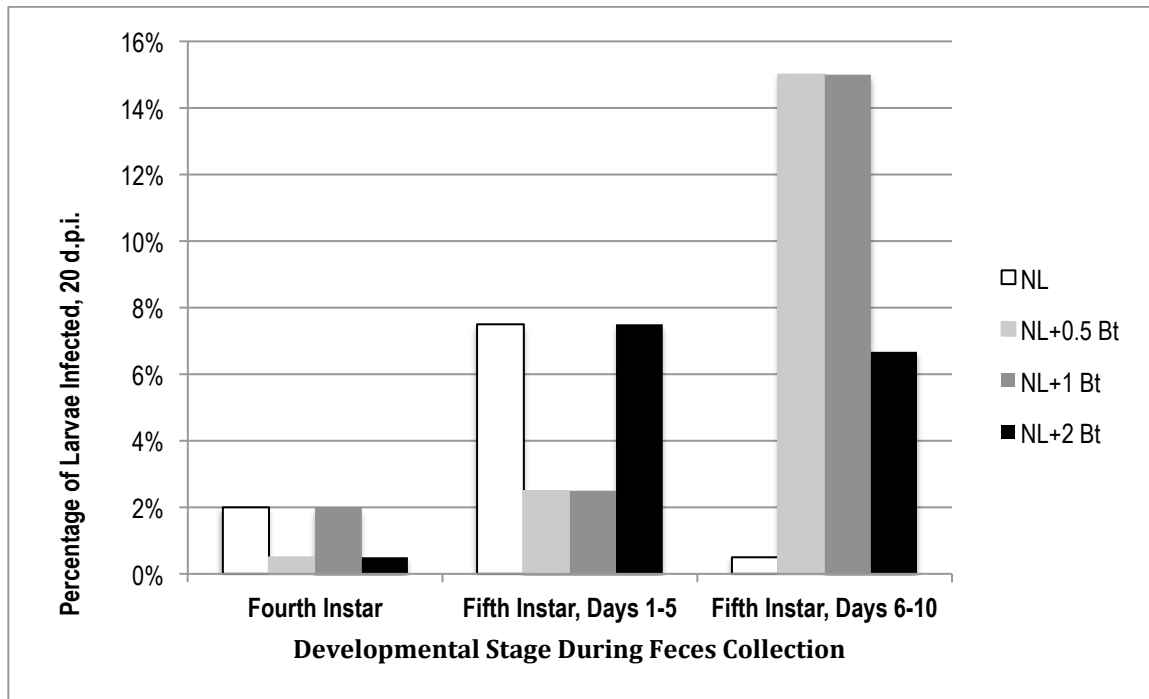
Fig. 5b Percentage of larvae successfully infected with spores derived from feces collected from larvae at different periods and of different mixed infection treatments, second trial. The lack of stars above bars indicates no significant differences among treatments within each spore collections phase.

3.5 Vertical Transmission

Incubated egg masses exhibited low hatching rates (Table 4). Of 7 egg masses derived from control larvae, only 4 hatched any larvae. Only three egg masses derived from a mother infected with *N. lymantriae* and Bt were incubated, but larvae emerged from only two egg masses. Of the unhatched eggs in the infected egg masses, 97-99% were fertilized. Egg fertilization rates for control egg masses were not recorded. The number of larvae infected with *N. lymantriae* increased with increasing Bt dose administered to the parent (Table 4). Mean hatch date and mean survival time were significantly later in two control egg masses (K22xK36 and K39xK34; One-way ANOVA, F = 97.202 and 43.166, d.f..= 5, *p*= 0,000) than in other control and infected egg masses.

Table 4 Number of larvae hatched (emerged) from each egg mass half and percentage of larvae infected with *N. lymantriae* spores (n_i= 30). Progeny derived from parents (left column) from a previous study. Parental treatments indicated as father x mother, where K= control (parent not infected), or NL= *N. lymantriae* + Bt dosage (0.25, 0.5, or 1 infection units), followed by a numerical identification. Statistical differences (One-way ANOVA, F = 97.202 (hatch) and 43.166 (survival), d.f..= 5, *p* < 0.001) indicated by different letters among egg mass treatments.

Maternal x Paternal Parent	No. Eggs Hatched Fed/Starved	% Larvae Infected	Mean Hatch Date [days]	Mean Survival Date [days]
K27 x K21	91/188	n/a	2.5 ± 0.5^a (188)	4.7 ± 0.5^a (188)
K25 x K35	188/32	n/a	2.7 ± 0.1^a (121)	4.5 ± 0.1^a (121)
K22 x K36	44/243	n/a	3.6 ± 0.5^b (243)	5.2 ± 0.5^b (243)
K39 x K34	40/176	n/a	4.1 ± 0.6^c (176)	5.7 ± 0.5^c (176)
NL+0.25 Bt 16 x K17	7/0	0%	0 (0)	0 (0)
NL+0.5 Bt 11 x K12	54/2	20%	2^a (2)	3.5 ± 0.5^a (2)
NL+1 Bt 27 x NL+1 Bt 27	101/26	73%	2.2 ± 0.1^a (26)	4.8 ± 0.2^{ab} (26)

4 Discussion

Sub-lethal Bt doses do not seem to conspicuously interact with *N. lymantriae* in gypsy moth. The data from both trials do not suggest interactive effects between sub-lethal Bt dosages and *N. lymantriae* spore counts in feces collected from fourth and fifth instar larvae, due to the lack of significant differences of mean spores among treatments and within collection phases, as well as the conspicuous overlap in ranges within and among collection phases in each trial. The differences between trials, however, are worthy of further consideration. Feces collected during the fourth instar of the first trial show considerable variation in amount of spores, including ranges comparable to subsequent collection phases, and a sample with the single highest spore concentration in the entire study (3.1×10^7 spores, NL+2 Bt). This contrasts to the data from the second trial, where means and ranges of spores in feces collected from the fourth instar were an order of magnitude lower than those in the first trial, and median concentrations were also considerably lower. Further, spore counts in second trial, fourth instar treatments exhibited conspicuously narrower ranges relative to later collection phases in the same trial, much unlike the overlapping ranges among collection phases in the first trial. These differences may, in part, be explained by the differences in lengths of the fourth instar between trials. Average lengths of fourth instars in all treatments of the first trial were longer (10.5-12.7 days) than fourth instars in the second trial (6.1-6.8 days). Because fourth instar feces were collected over a developmentally-defined time period rather than a fixed number of days (as in the five day intervals for collection in the fifth instar), more feces, and therefore spores, would have been collected if the fourth instar lasted longer. Additionally, the range of the end of the fourth instar among treatments in the first trial (17.7-25.5 dpi) overlaps the period of first occurrence of spores in feces (12-19 dpi) observed by Goertz and Hoch (2008a) more so than the range of the end of fourth instars in the second trial (11.9-14.5 dpi) that more overlaps spore latent period, allowing for the *N. lymantriae* infections to become more systemic and to produce more spores in feces. The prolonged fourth instar in the first trial resulting in possible infection-related differences in larval development (Pollan 2009) might explain the lower percentage of larvae that survived molting to the fifth instar (7-47%) relative to the second trial (84-100%) due to increased time allowed for micropsoridian infection to progress. Collection of feces at fixed time intervals in the fourth instar might help clarify these observations.

Spores counts in L5 1-5 and L5 6-10 feces were also substantially different between trials, with mean, medians, and ranges 20-88% higher in the first trial. These differences may again be partially attributable to the earlier onset of the fifth instar in the second trial, which may have affected the timespan for proliferation of the microsporidia infection and ultimate rate of spore excretion into feces. The total amount of spores released from NL-only treatments during the fourth instar and ten days of the fifth instar were 3.3×10^7 spores for the first trial, and 1.0×10^7 spores for the second trial, are approximately comparable to the total spore count previously reported for *N. lymantriae* in feces ($2.7 \times 10^7 \pm 8.1 \times 10^6$) in Goertz and Hoch (2008a).

The spore counts and mortality data support the findings of Mayrhofer (unpublished) study of dual infections and spore content of cadavers. At sub-lethal Bt dosages, *N. lymantriae* is the

sole cause of death in larvae inoculated with both pathogens; all *N. lymantriae*–inoculated individuals that died before eclosion exhibited conspicuous microsporidiosis when dissected. An unexpected finding in both trials of this study, however, was the lack of Bt-induced mortality in Bt-only and mixed infection larvae. The range of Bt-doses for this study were selected to span sub-lethal (0.5 IU and 1 IU Bt) and minimally lethal (2 IU Bt) concentrations based on an LD_{50} of 1.95 IU from Mayrhofer (unpublished) and van Frankenhuyzen *et al.* (2008), but all Bt-only inoculated larvae in this study survived to pupation. The delayed development during the third and fourth instars observed at higher Bt concentrations in both trials suggests that the Bt inoculations in this study were conducted properly, and are similar to reported effects of sub-lethal Bt doses (Erb *et al.* 2001), though Erb *et al.* (2001) also reported differences in male pupal weight, and Bt inoculations were conducted by force feeding at the onset of the fourth instar. This study utilized a different bottle of Bt (albeit same lot number and age) Btk than Mayrhofer (unpublished). A preliminary bioassay comparing the effectiveness of the two bottles demonstrated that larvae inoculated with the former exhibited somewhat delayed mortality (median death: 4 dpi for 16 IU and 3.5 dpi for 32 IU) compared to the latter (median death: 3 dpi at 16 and 32 IU), which may suggest differential variability in effectiveness between bottles that might account for the low Bt-mediated lethality observed in this study.

The absence of apparent effects of Bt on microsporidia spore counts in feces is consistent with Mayrhofer (unpublished) findings in cadavers, where larvae infected with NL+0.5 IU Bt and NL+1 IU Bt exhibited similar number of spores/mg larval fresh mass to NL-only control treatments. Mayrhofer (unpublished) did show decreased spore content in NL+2 IU Bt treatments, unlike the present study, but this is likely attributable to the aforementioned shift in the lethal/sub-lethal Bt dose threshold observed between the two studies. These studies contrast to Pierce *et al.* (2001), where increasing Bt dosages reduced spore counts in *Ostrinia nubilalis* Hubner larvae at all tested doses, instars, and exposure levels. However, Bt doses in this study were administered in continuous and lethal doses.

Nonetheless, the absence of Bt effects on *N. lymantriae* spore counts is not wholly unexpected. There is currently no *a priori* known mechanism by which sub-lethal Bt doses would necessarily alter apparent spore counts in larvae or feces. In principal, since midgut cells damaged by Bt are shed after infection (Hakim *et al.* 2010), a sufficient number of shed *N. lymantriae*-infected midgut cells could reduce the amount of microsporidia spores in the initial midgut incoculum, which might, in-turn, decrease the number or rate of infection of target tissues by primary spores released from midgut cells. But as Mayrhofer (unpublished) demonstrated, the doses of Bt that might cause such sufficient damage to reduce the rate of microsporidia spread from midgut to target tissues ultimately kill the larvae.

The effect of sub-lethal Bt concentrations on the viability of spores collected from feces is still not understood. Both attempts resulted in far lower rates of infections in all treatments than expected. Spores derived from NL-treatments particularly should have exhibited substantially higher infection rates. Whereas Goertz and Hoch (2008a) observed 0%, 100%, 20%, and 93% infectivity at 6-10, 11-15, 16-20, and 21-25 dpi, respectively, NL-only-derived spores in this study exhibited infectivity of 5-35% in the first trial and 0-8% infectivity in the second trial at approximately similar collection periods. Coupled with the absence of significant differences

among treatments, discernment of any effects Bt might have on spore viability becomes unfeasible. The failure of both trials suggests methodological or systematic errors during the experiment. The only recognized methodological differences between this study and the successful viability experiment of Goertz and Hoch (2008a) was the container and frequency of feces collection. Goertz and Hoch (2008a) reared larvae in ventilated 50 ml plastic diet cups and collected feces from these cups every five days after infection. In this study, larvae were reared in 9 x 1 cm petri dishes and feces were collected at less regular and potentially longer intervals in the fourth instar. If the petri dishes were subjected to longer intervals at higher humidity relative to the diet cups, this would promote the growth of bacteria and fungi in the feces that might damage or otherwise reduce the viability (and therefore infectivity) of *N. lymantriae* spores in those feces. Alternatively, feces and extracted spores may have been stored in a freezer that was not sufficiently cool or did not freeze samples fast enough potentially resulting in excessive ice crystal formation and damaged microsporidian spores with reduced viability. *N. lymantriae* spores have demonstrated sensitivity to *in situ* low temperature conditions, with spores in cadavers showing much higher viability when overwintered under snow cover than when cadavers are left exposed on tree trunks or on the ground without snow cover (Goertz and Hoch 2008b). However, most spores collected for the second trial were stored with added glycerol to reduce ice crystal formation, but this did not positively affect observed spore infectivity.

Discerning meaningful patterns from the vertical transmission study is obscured by limited sample sizes. During the course of the experiment, a second set of control egg masses were incubated to compensate for the failure of an initial set of control egg masses (not shown) to hatch. Additionally, several egg masses were found to contain dozens of larvae that appeared to have prematurely hatched during storage (after eggs were laid but prior to incubation). This suggests egg masses may have been under cold storage too long or were put in cold storage too late. Some starved control egg masses showed significantly greater m hatch and survival times, but other starved control egg masses showed hatch and survival times comparable to infected egg masses. Mixed infection therefore does not seem to affect these parameters. The increasing rate of infectivity with increasing maternal Bt exposure, though highly speculative given the limited sample size, may be worth further investigation.

Elucidating the interactions between Bt and *Nosema lymantriae* or other microsporidia pathogens in gypsy moth are critical for understanding the potential effects of widespread Bt use on natural microsporidia outbreak patterns in Europe, as well as for examining the potential of microsporidia as augmentative and biological control agents. This study, along with the Mayrhofer (unpublished) demonstrated that the effects of mixed *Bt/N.lymantriae* infection depend on Bt concentration. At lethal doses, Bt causes larval mortality before *N. lymantriae* infection can become systemic, therefore inhibiting horizontal and vertical transmission and thus its usefulness as a regulator of gypsy moth outbreaks and/or as a biocontrol agent. At sublethal doses however, these studies indicate Bt does not negatively impact spore concentration in cadavers and feces, the two principal horizontal transmission pathways. Theoretically, Bt could therefore be applied at a different time than augmentative or classical biological control applications of *N. lymantriae*, but not simultaneously and not during natural microsporidia outbreaks. If the methodological problems encountered here can be resolved, however, further studies examining the effects of Bt on microsporidian spore viability are essential for a more

complete understanding of Bt-microsporidia interactions. Additional studies involving more egg masses would help clarify the effects of Bt on vertical transmission, though this requires perhaps unmanageably large numbers of parents due to the high mortality of *N. lymantriae* infected insects. Finally, more thorough studies on the developmental impacts of mixed infections would expand and explain the developmental delays in mixed-infection larvae observed at higher sub-lethal Bt doses.

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6 Index of Tables and Figures

7 Appendix

One-way ANOVA statistical output parameters

Table A-1 One-way ANOVA parameters for Bt treatments, first trial. Parameters separated female/male.

Treatment	F	d.f.	μ^2	p
Wt. 1 dpi	3.351/0.551	3	0.000/0.000	0.320/0.653
Wt. death	n/a	n/a	n/a	n/a
Wt. pupae	0.307/0.650	3	0.095/.031	0.820/0.591
Dpi 4th Instar	6.706/3.185	3	12.544/8.742	0.001/0.044
Dpi.5th Instar	4.247/0.424	3	8.570/1.156	0.014/0.741
Dpi. pupation	1.037/1.266	3	15.522/16.858	0.392/0.312
Dpi adult eclosion	0.799/0.707	3	38.128/5.159	0.506/0.559
Dpi death	n/a	n/a	n/a	n/a

Table A-2 One-way ANOVA parameters for mixed infection treatments, first trial.

Treatment	F	d.f.	μ^2	p
Wt. 1 dpi	0.996	3	0.000	0.402
Wt. death	1.200	3	0.051	0.318
Wt. pupae	n/a	n/a	n/a	n/a
Dpi 4th Instar	6.449	3	10.333	0.001
Dpi.5th Instar	0.952	3	2.163	0.447
Dpi. pupation	n/a	n/a	n/a	n/a
Dpi adult eclosion	n/a	n/a	n/a	n/a
Dpi death	1.686	3	18.194	0.180

Table A-3 One-way ANOVA parameters for Bt treatments, second trial. Parameters separated female/male.

Treatment	F	d.f.	μ^2	p
Wt. 1 dpi	1.162/0.210	3	0.000/0.000	0.359/0.888
Wt. death	n/a	n/a	n/a	n/a
Wt. pupae	0.426/0.637	3	0.172/0.004	0.738/0.603
Dpi 4th Instar	6.532/2.472	3	3.165/2.003	0.005/0.104
Dpi.5th Instar	2.506/4.478	3	4.483/8.343	0.101/0.021
Dpi. pupation	3.189/3.135	3	15.937/10.984	0.057/0.059
Dpi adult eclosion	6.231/2.916	3	24.900/8.714	0.017/0.078
Dpi death	n/a	n/a	n/a	n/a

Table A-4 One-way ANOVA parameters for mixed infection treatments, second trial.

Treatment	F	d.f.	μ^2	p
Wt. 1 dpi	2.693	3	0.000	0.054
Wt. death	1.608	3	0.351	0.195
Wt. pupae	n/a	n/a	n/a	n/a
Dpi 4th Instar	28.372	3	23.903	0.000
Dpi.5th Instar	19.920	3	26.464	0.000
Dpi. pupation	n/a	n/a	n/a	n/a
Dpi adult eclosion	n/a	n/a	n/a	n/a
Dpi death	3.570	3	95.435	0.017