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Incubation period, spore egestion and horizontal transmission of *Nosema fumiferanae* (Microsporidia: Nosematidae) in spruce budworm (*Choristoneura* sp., Lepidoptera: Tortricidae): The role of temperature and dose

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Abstract

Various instars of *Choristoneura occidentalis* were fed with a range of doses of *Nosema fumiferanae* and reared at 20, 24 and 28 °C to determine the influence of temperature and dose on the time to spore egestion and the number of spores egested in the frass. When larvae were fed in the third stadium, as few as 10^2 spores per larva initiated infection, and both onset of spore egestion and the number of spores egested were affected by a complex relationship between temperature and inoculation dose. Onset of spore egestion varied from 11 to 15 days postinoculation. At 20 °C, the onset was delayed and spore production decreased with increasing inoculation dose whereas at higher temperatures spores were first egested at the lowest dose and spore production increased with dose. When larvae were fed spores in the fifth and sixth stadium, no spores were egested because pupation occurred before completion of the incubation period. To assess the effect of temperature on horizontal transmission, *Choristoneura fumiferana* larvae fed with 10^4 *N. fumiferanae* spores per larva were reared with uninfected larvae at 15, 20 and 25 °C. At 15 °C, we observed the highest degree of horizontal transmission, defined by the largest change in *N. fumiferanae* prevalence, even though the density of spores available for horizontal transmission was the lowest. Infected adults eclosed later than uninfected adults and the time to eclosion was also dependent on sex and temperature. We relate our experimental findings to consequences for horizontal and vertical transmission of *N. fumiferanae* in spruce budworm populations.

Keywords: *Choristoneura fumiferana*; *Choristoneura occidentalis*; Spruce budworm; Microsporidia; *Nosema fumiferanae*; Horizontal transmission; Temperature; Dose

1. Introduction

Horizontal transmission is a common mechanism for the spread of disease in forest Lepidoptera populations that can result in second-order density dependent regulation (Dwyer, 1994). Successful horizontal transmission has two components: (1) encounter of pathogen propagules in the environment and (2) initiation of infection after encounter (Knell et al., 1998). The probability of healthy individuals encountering pathogen propagules depends on the host's life history, behavior and susceptibility to the pathogen (Knell et al., 1998; Hajek, 2001), the density of uninfected larvae (Dwyer, 1991), and the density of propagules in the environment (Onstad and Maddox, 1989; Reeson et al., 2000). The latter depends to a large extent on the density of infected hosts (Knell et al., 1996; D'Amico et al., 1996; Dwyer et al., 1997; Siegel et al., 1988; Fenton et al., 2002).

Nosema fumiferanae (Thompson) is a protozoan parasite of the spruce budworm, *Choristoneura fumiferana* (Clem.) (Thomson, 1955, 1958a) and other budworm species

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(Thomson, 1958b), which can reach high prevalence levels during outbreaks of its host (Wilson, 1987). While infection is typically sublethal (Thomson, 1958a,b; Bauer and Nordin, 1988a), host mortality does occur at high infection levels (Bauer and Nordin, 1988b; Wilson, 1985). *N. fumiferanae* may play a critical role in regulating spruce budworm populations (Régnière, 1984). Horizontal transmission spreads *N. fumiferanae* among larvae within a generation and determines the number of infected females capable of transmitting infection to their offspring. Factors to be considered when examining horizontal transmission include the density of spores egested into the environment by infected individuals that are available for ingestion by other larvae, and the number of *N. fumiferanae* spores required to give rise to new infection. The younger the larva, the fewer spores are needed to establish infection (Wilson, 1974).

To initiate infection, *C. fumiferanae* larvae must ingest mature *N. fumiferanae* spores (Thomson, 1958a). Once in the midgut, spores extrude a polar tube that penetrates the cells. Sporoplasm passes through the polar tube and into the midgut cell. Initial replication of the pathogen produces spores that spread infection from cell to cell in the midgut and other tissues such as the fat body. Mature spores are produced within midgut cells and upon cell lysis are passed into the lumen of the gut and are egested with the frass.

We examined some key questions related to horizontal transmission of *N. fumiferanae* in spruce budworm. We focused on host–pathogen encounters and the number of spores required to give rise to infection. We determined the time between spore ingestion and the first appearance of spores in the frass (hereafter referred to as incubation period) as a function of inoculation dose, temperature and instar. We monitored the accumulation of spores egested in the frass over time (hereafter referred to as egested spore production) as a function of temperature and inoculation dose. We also examined the effect of temperature on horizontal transmission among larvae in artificial feeding arenas.

2. Methods

2.1. Insects

Overwintered second instar *C. fumiferana* larvae were obtained from the Insect Production Unit at the Great Lakes Forestry Center of the Canadian Forest Service in Sault Ste. Marie, Ontario. Larvae were reared according to protocols described by Grisdale (1984) on artificial diet without antimicrobial agents. The food was changed every seven days. All larvae were reared at 20 °C ± 1 °C and 16-h light/8-h dark, unless stated otherwise. Larvae used in all experiments had molted to the desired instar within the previous 24 h. To determine the incubation period and egested spore production, *C. occidentalis* was used because sufficient *C. fumiferana* larvae were not available. *N. fumiferanae* readily infects *C. occidentalis* and we routinely use a nondiapause strain of that species as a substitute for *C. fumiferana* to produce spores.

2.2. Microsporidia

Nosema fumiferanae spores were obtained from *C. fumiferana* larvae collected from field populations in Ontario and Quebec in 2002 and 2003. Spores were harvested from infected larvae as described by Van Frankenhuyzen et al. (2004), purified with Ludox (Undeen and Alger, 1971) and stored in liquid nitrogen in 50% glycerol. Before each trial, the glycerol was removed from the spore suspensions by repeated washes with filter-sterilized water (fsH_2O) and centrifugations (3×15 min) at 3200 rpm. The pellet was resuspended in fsH_2O to a volume of 1 ml. Spore concentrations were determined using a haemocytometer. Dilution series were established and confirmed by using a haemocytometer and were stored overnight at 4 °C.

2.3. Incubation period and egested spore production

Third-instar (L3) *C. occidentalis* larvae were fed (as per Van Frankenhuyzen et al., 1997) with 10^2, 10^3 or 10^4 spores per larva and fifth and sixth instar larvae (L5 and L6) were fed with 10^5 or 10^6 spores per larva. Control larvae were fed with fsH_2O. Only larvae that ingested the entire droplet were used. Exposed larvae were reared individually at 20, 24 or 28 °C in 25-ml creamer cups. A total of 105 larvae were thus obtained for each combination of dose, temperature and instar.

For each treatment, frass was collected each day from five different larvae so that frass production was measured for all 105 larvae on a 7-day cycle because spore production is variable between individual larvae (Wilson, 1985). We chose a 7-day measurement interval for individual larvae based on preliminary estimates of the incubation period. Frass collections were extended to 16 days postinoculation to cover the entire incubation period in all treatments. The frass and silk strands containing entangled frass pellets were collected with a camel hair brush or fine forceps that were washed in 70% ethyl alcohol between collections.

Frass samples were homogenized in an equal volume of fsH_2O and were examined under the microscope to determine: (1) the time of first appearance of *N. fumiferanae* spores in the frass, and 2) the number of spores egested in the frass. Wet mounts of frass homogenates (10 µl) were examined for the presence of spores using phase contrast at 400× magnification. Spores were counted with a haemocytometer for homogenates from days 11 to 16 postinoculation. Frass collection among larvae exposed to *N. fumiferanae* at L5 and L6 proceeded until pupation (8–12 days postinoculation).

2.4. Horizontal transmission

Third-instar *C. fumiferana* larvae were fed with 10^4 spores of *N. fumiferanae* per larva and reared at 15, 20 and 25 °C (500 larvae per temperature). Another 300 uninfected larvae were individually reared at each of the three temperatures. After 10 days, groups of 12 larvae (six uninfected

and six exposed) were placed together in 225-ml cups containing artificial diet (surface area $\approx 64\,cm^2$) and reared at 15, 20 or 25 °C (15 replicates) plus one control cup each with all exposed or all uninfected larvae. At that time, the majority of larvae were early L4s at 15 °C, early L5s at 20 °C, and early L6s at 25 °C. The diet was changed regularly until pupation to prevent desiccation. Pupae were sexed, transferred to individual cups and reared at the corresponding treatment temperature. Time to adult eclosion from the onset of L3 and mortality during the larval and pupal stage were recorded.

Microscopy was used to determine prevalence of *N. fumiferanae* in larvae at the onset of the experiment (initial prevalence) and in adults at the end (final prevalence). To determine initial prevalence, a subsample of 50 exposed larvae was selected after the 10-day rearing period preceding assignment to the various treatments. Each larva was homogenized in 200 µl of fsH_2O and 5 µl of each homogenate was smeared, stained with Naphthalene black 12B (Evans and Shapiro, 1997) and examined microscopically at 400× magnification. To determine final prevalence, the abdomens of first day adults were homogenized in 400 µl of fsH_2O, smeared, stained and examined at 400× magnification.

To determine the amount of spores available for horizontal transmission, frass was collected from each cup after all larvae had pupated. Frass samples were homogenized in an equal volume of fsH_2O and spore counts were conducted with a haemocytometer, using phase contrast at 400×.

2.5. Statistical analysis

First appearance of spores in the frass was used as an indication that one complete replication cycle of *N. fumiferanae* had occurred (incubation period). Egested spore production was determined by summing total spores recovered in the frass for each individual. Larvae that died before pupation were not used in the analyses. Binary logistic regression (Pagano and Gauvreau, 2000) was used to determine the effect of temperature and initial *N. fumiferanae* dose on incubation period (presence of spores, PROC GENMOD/OFFSET = *n*(sample day), SCALE = Pearson, SAS Institute Inc., 1999). Poisson regression was performed to determine the effect of temperature and initial *N. fumiferanae* dose on egested spore production through time (number of spores, PROC GENMOD/OFFSET = ln(sample day), SCALE = Pearson, SAS Institute Inc., 1999). It was not possible to test the interaction between temperature and dose because there were not enough degrees of freedom as a result of saturated regression models. Analysis of variance (ANOVA) could not be used because residuals failed to meet assumptions of equal variances and normality.

Spore presence was used to determine the initial and final proportion of larvae infected with *N. fumiferanae* (i.e. the initial and final *N. fumiferanae* prevalence). Cups containing more than four missing larvae, those cups with more individuals than initially added and cups with obvious fungal growth on the food were removed from all analyses. Binary logistic regression was used to determine the effect of temperature on initial and final prevalence (Minitab Inc., 2004). A general linear ANOVA was performed to assess the relationship between temperature, time and dose on egested spore production (Minitab Inc., 2004), and to determine the effect of sex, infection status and temperature on the time to adult eclosion from the L3 stadium. The three-way interaction and the sex-infection interaction were removed from the model because they had no significant effect on the mean time to eclosion.

3. Results

3.1. Incubation period

Spores did not appear in the frass of larvae exposed to *N. fumiferanae* as third-instars until 11 days after inoculation (Table 1). The incubation period was dependent on temperature ($\chi^2 = 14.89$, df = 2, $P < 0.001$) but not on initial dose ($\chi^2 = 4.47$, df = 3, $P = 0.215$). As few as 10^2 spores per larva initiated infection. At that dose, spores appeared 11–12 days after inoculation when larvae were reared at 20 or 24 °C, in contrast to 15 days at 28 °C. When larvae were exposed to 10^3 and 10^4 spores per larva, the first egested

Table 1
Incubation period of *N. fumiferanae* in third-instar *C. occidentalis* larvae: Proportion of frass samples containing *N. fumiferanae* spores

Treatment			Days after initial inoculation[a](*n*)				
Dose (spores per larva)	Temperature (°C)	Cumulative % (mortality)	11	12	13	14	15
10^2	20	1.72	0.00 (9)	0.20 (10)	0.10 (10)	0.67 (9)	0.33 (3)
	24	0.00	0.11 (9)	0.20 (10)	0.00 (10)	0.14 (7)	0.40 (5)
	28	2.04	0.00 (10)	0.00 (10)	0.00 (9)	0.00 (10)	0.30 (10)
10^3	20	0.00	0.00 (10)	0.00 (10)	0.00 (18)	0.00 (1)	0.20 (10)
	24	0.00	0.00 (9)	0.18 (11)	0.20 (10)	0.22 (9)	0.30 (10)
	28	6.00	0.00 (10)	0.22 (9)	0.20 (10)	0.55 (11)	0.40 (10)
10^3	20	3.40	0.00 (8)	0.00 (10)	0.00 (10)	0.00 (11)	0.11 (9)
	24	6.45	0.00 (10)	0.56 (9)	0.44 (9)	0.18 (11)	0.79 (14)
	28	14.81	0.00 (10)	0.08 (12)	0.00 (10)	0.11 (9)	0.69 (13)

[a] The length of time between inoculation and the time of frass collection.

spores were encountered after 12 days at 24 and 28 °C but not until 15 days at 20 °C. Inoculation with 10^2 and 10^3 spores per larva caused little mortality; the highest mortality (15%) occurred among larvae exposed to 10^4 and reared at 28 °C. Spores did not appear in the frass of larvae exposed as late instars regardless of dose or rearing temperature. Measurements were stopped at pupation (L5: up to 12 days after inoculation, $n = 54$; L6 up to 8 days after inoculation $n = 84$).

3.2. Egested spore production

Egested spore production increased through time in a temperature- and dose-dependent fashion (Fig. 1). At 10^2 spores per larva, the cumulative egested spore production was highest at 20 °C and lowest at 28 °C, reflecting the shorter incubation period noted above. When larvae were dosed with 10^3 spores per larva, the highest spore production occurred at 28 °C, and with 10^4 spores per larva the maximum occurred at 24 °C. Thus, the rate of egested spore production was affected by temperature ($\chi^2 = 20.46$, df = 2, $P < 0.001$) and by initial dose ($\chi^2 = 16.14$, df = 3, $P = 0.001$), as well as by their interaction (Fig. 1).

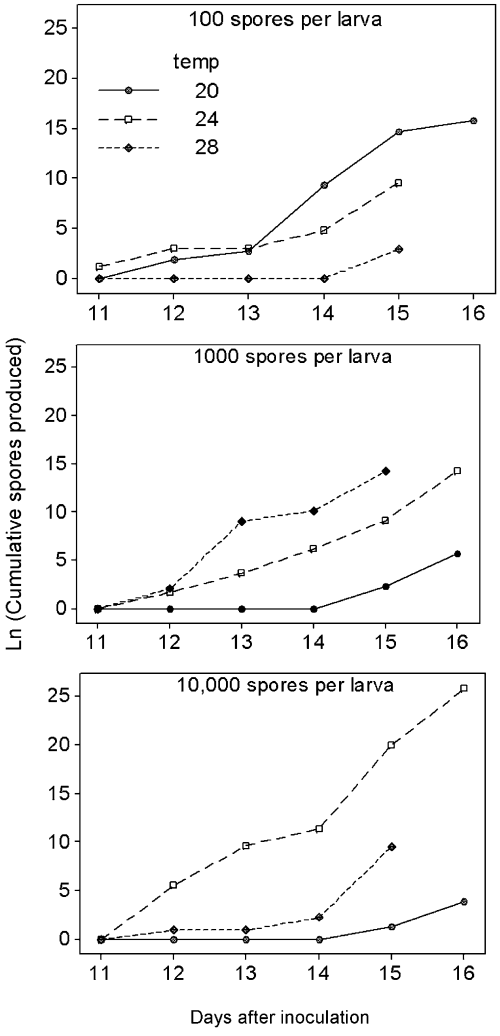

Fig. 1. Cumulative number of spores egested in the frass when *Choristoneura occidentalis* larvae were fed as third-instar with *Nosema fumiferanae*. The Ln (cumulative number of spores) is represented as a function of rearing temperature (°C) and initial *N. fumiferanae* inoculum (spores/ larva).

3.3. Horizontal transmission

Neither initial nor final proportion of larvae infected with *N. fumiferanae* was significantly affected by temperature (Fig. 2a; initial prevalence: $\chi^2 = 3.45$, df = 2, $p = 0.178$; final prevalence: $\chi^2 = 0.036$, df = 2, $P = 0.982$). The highest degree of horizontal transmission (final–initial prevalence) occurred at 15 °C (0.122) and the lowest at 20 °C (0.065). Although larval mortality was highest at 15 °C (Table 2), larval death did not affect final *N. fumiferanae* prevalence ($\chi^2 = 0.11$, df = 2, $P = 0.946$).

The production of spores available for horizontal transmission was affected by temperature ($F = 8.04$, df = 2, 42, $P = 0.001$) (Fig. 2b). Although the observed degree of possible horizontal transmission was the greatest at 15 °C, the density of egested spores was lowest at that temperature.

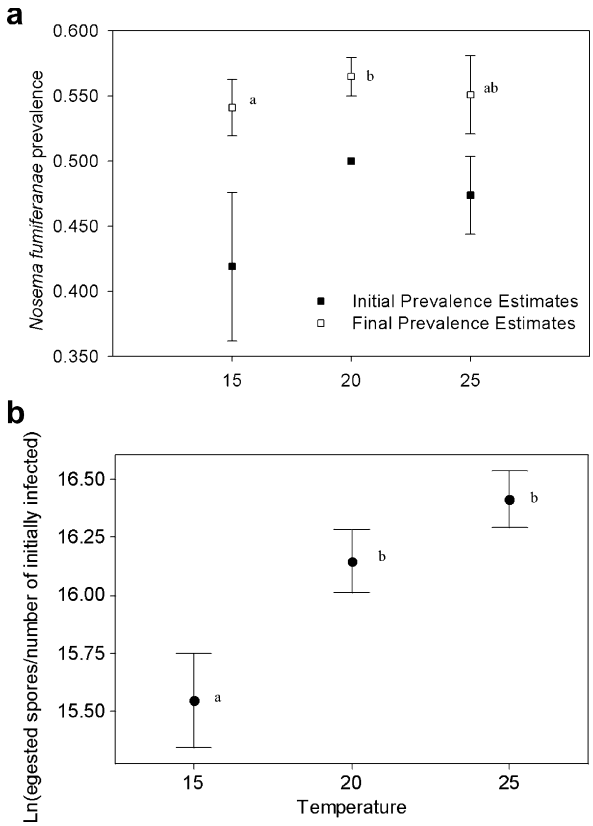

Fig. 2. (a) Initial and final proportion of *N. fumiferanae* infected larvae (prevalence) estimated from horizontal transmission experiments (Initial Estimates: n (all temperatures) = 50; Final estimates: n (15 °C) = 113, n (20 °C) = 152, n (25 °C) = 134). Common letters represent insignificant differences in the change of prevalence (t-test). (b) Number of spores (natural log transform) present in the frass available for horizontal transmission at each temperature. Bars represent one standard error of the mean and common letters represent insignificant differences (t-test).

Table 2
Days to adult eclosion ± SE of *C. fumiferana* in the horizontal transmission experiment as a function of temperature (°C), sex and *N. fumiferanae* infection status

Temperature (°C)[a]	Number of replicates[b]	Number of larvae[c]	Number of lost[d]	Number of dead[e]		Number of adults enclosed	Sex	*Nosema* infected?	Mean days to adult eclosion	*n*
				Larvae	Pupae					
15	12	144	15 (10.41)	16 (12.40)	34 (25.95)	79	Male	Uninfected	45.75 ± 0.80	16
							Male	Infected	48.27 ± 0.96	26
							Female	Uninfected	47.04 ± 0.44	24
							Female	Infected	52.77 ± 1.31	13
20	14	168	7 (4.17)	9 (5.59)	26 (16.15)	126	Male	Uninfected	25.13 ± 0.23	30
							Male	Infected	27.72 ± 0.40	43
							Female	Uninfected	27.10 ± 0.31	28
							Female	Infected	28.68 ± 0.54	25
25	13	158	12 (7.59)	12 (8.45)	5 (3.52)	129	Male	Uninfected	18.97 ± 0.25	31
							Male	Infected	19.70 ± 0.33	40
							Female	Uninfected	20.07 ± 0.18	29
							Female	Infected	21.86 ± 0.36	29

[a] Larvae were dosed with 10^4 *N. fumiferanae* spores per larva and reared with uninfected larvae at three temperatures.
[b] Number of rearing cups per temperature; each cup contained six inoculated and six uninfected larvae.
[c] Total number of larvae at the start of the experiment in each temperature treatment.
[d] Number of larvae lost during the experiment (escape of small larvae) (percent loss).
[e] Number of dead larvae and pupae (percent mortality).

Table 3
Effect of temperature, sex and infection status on the length of time to adult eclosion of *C. fumiferana* larvae in the *N. fumiferanae* horizontal transmission-temperature experiment, modeled by a general linear ANOVA ($R^2 = 94.89$)

Source	df	*F*	*P*-value
Temperature	2	4072.67	<0.001
Sex	1	86.40	<0.001
Nosema Infection	1	103.35	<0.001
Temperature × Nosema Infection	2	25.34	<0.001
Temperature × Sex	2	14.53	<0.001
Error	447		
Total	455		

3.4. Adult eclosion

Fewer adults eclosed and eclosion was delayed at 15 °C in comparison with the other temperatures (Table 2). At 25 °C, mortality was lowest, more adults eclosed and eclosion was more rapid. At each temperature, infected adults eclosed later than uninfected adults and females eclosed later than males. The mean time to eclosion was affected by temperature, *N. fumiferanae* infection, sex, and by infection-temperature and sex-temperature interactions (Table 3).

4. Discussion

For successful horizontal transmission, the host must encounter disease propagules in the environment. The density of the propagules at a given time depends largely on two factors: (1) the incubation period (the delay between initial infection and release of propagules into the environment) and (2) the number of propagules released.

Spruce budworm larvae that were exposed to spores of *Nosema fumiferanae* near the end of their larval development (L5, L6) did not release spores in the environment by egestion as they pupated before the end of the incubation period. In contrast, larvae infected as third instars egested spores within 11 to 15 days after initial inoculation (Table 1). The incubation period in L3 varied with temperature, but this relationship was strongly affected by inoculation dose. At 20 °C, larvae fed with 10^3 or 10^4 spores started egesting spores three days later then those fed with 10^2 spores. Yet at the two higher temperatures (24 and 28 °C) spores first appeared in the frass from larvae exposed at the lower rate. Bauer and Nordin (1988a) observed that inoculation dose of *N. fumiferanae* influences the proliferation of the disease rather than its ability to initiate infection. It is also known that proliferation of microsporidia occurs faster at lower temperatures than at higher temperatures compared to the development rate of the host (Solter et al., 1990). Moreover, infected larvae have a reduced ability to convert ingested food to body mass (Bauer and Nordin, 1988b). We postulate that the level of infection resulting from the lowest dose (10^2) caused little stress on food utilization in the slowly developing host larvae at 20 °C, which allowed for a more rapid proliferation of the disease and resulted in earlier egestion of spores compared to the higher doses. At 28 °C, more rapid larval development could have outpaced the proliferation of *N. fumiferanae*, causing the delay in egested spore appearance compared to higher doses. At 24 °C spores appeared within 12 days regardless of dose indicating that this temperature is likely an optimum trade off between disease proliferation and larval development.

The quantity of spores released by infected larvae is an important factor in the development of epizootics.

Wilson (1974, 1978, 1985) demonstrated a parabolic relationship between *N. fumiferanae* spore yield in spruce budworm larvae and temperature with an optimal yield at 23 °C. We expected similar results for egested spore production. However, we found a complex relationship between spore production and temperature that was modulated by inoculation dose (Fig. 1) and that mirrored the relationship observed for the incubation period. The lowest levels of spore production (Fig. 1) corresponded to the longest incubation periods (Table 1), most likely for the same reasons as discussed above.

Our results indicate that spruce budworm larvae have to be infected early in their development in order to contribute spores for horizontal transmission. Only the larvae infected as third-instars egested spores into the environment. Larvae infected as L5 and L6s did not egest any spores and can therefore only contribute to vertical transmission as adults. In the field, horizontal transmission typically occurs during the sixth larval stadium when larvae search for new feeding sites (Thomson, 1958a), but may occur earlier when high larval densities force earlier dispersal from depleted feeding sites.

Results from our laboratory transmission experiment suggest that when spores are readily accessible in the environment and the length of time for spore encounter is long, spore encounter may be a more important determinant for horizontal transmission than spore density. The dose ingested appears to be of little consequence to horizontal transmission among spruce budworm larvae, at least under our experimental conditions, since as little as 10^2 spores per larva initiated infection (Table 1) and the greatest horizontal transmission occurred at the lowest spore density (Fig. 2). We suspect that slow larval development at 15 °C compensated for the low spore production by allowing more time for uninfected larvae to encounter egested spores. A field experiment was undertaken to examine horizontal transmission as a function of dose and larval density under natural conditions, but it failed because the *C. fumiferana* larvae used in that experiment were, unbeknownst to us, infected with a more virulent microsporidian (Van Frankenhuyzen et al., 2004) that outcompeted *N. fumiferanae*.

Nosema fumiferanae infection delayed adult eclosion at each temperature. Such a delay is unlikely to affect transmission of the disease because similar proportional delay in development of both sexes means that infected females are most likely to mate with infected males, and mating success is not adversely affected by infection (Thomson, 1958a).

Our reported 11–15 day incubation period differs from the 7-day replication cycle of the microsporidian pathogens of *Lymantria dispar* (Solter et al., 2002) but is similar to that of *Nosema whitei* in *Tribolium confusum* (Onstad and Maddox, 1990). The extended incubation period of *N. fumiferanae* might be explained by: (1) tissue specificity of infection, (2) differences in virulence (as per Shapiro Ilan et al., 2005) between microsporidian species, or (3) virulence differences between lethal and sublethal pathogens. *N. fumiferanae* infects all tissues of the spruce budworm (Thomson, 1955) while microsporidia in the gypsy moth are restricted to specific tissues (Solter et al., 2002). The lower degree of *Nosema fumiferanae* tissue specificity may account for delayed spore egestion. Day (2003) suggested that less virulent pathogens have slower replicative cycles than pathogens with higher virulence. Thus, it is possible that *N. fumiferanae* is less virulent to its host than are gypsy moth microsporidia to their host. Furthermore, pathogens depending solely on horizontal transmission tend to have higher virulence with shorter incubation periods than pathogens relying on both horizontal and vertical transmission (Frank, 1996).

Little information is available on the transmission of chronic infections in insects, and further studies on the horizontal transmission of *N. fumiferanae* in the field are needed. Our study shows that the interactions between factors affecting horizontal transmission are complex. Future work should focus on the influence of density of both infected and susceptible larvae on horizontal transmission dynamics, in particular to validate horizontal transmission parameters that underpin many models of insect–pathogen dynamics. Additionally, unraveling the role of vertical transmission is necessary to understand the possible role of *N. fumiferanae* in the regulation of spruce budworm populations.

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