Biological Control 63 (2012) 164–171

Contents lists available at SciVerse ScienceDirect

Biological Control

journal homepage: www.elsevier.com/locate/ybcon

Biological and behavioral parameters of the parasitoid *Cotesia flavipes* (Hymenoptera: Braconidae) are altered by the pathogen *Nosema* sp. (Microsporidia: Nosematidae)

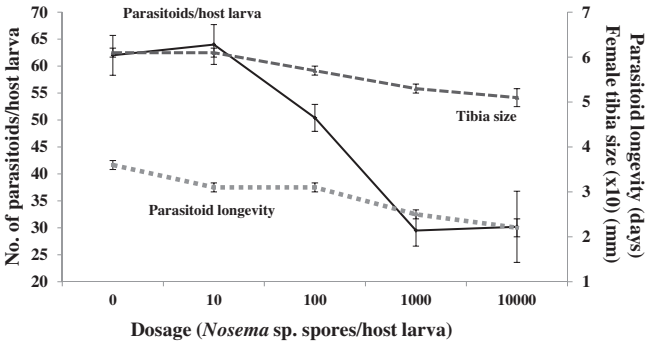
Renata A. Simões [a], Letícia G. Reis [a], José M.S. Bento [a], Leellen F. Solter [b], Italo Delalibera Jr. [a,*]

[a] Escola Superior de Agricultura "Luiz de Queiroz"- University of Sao Paulo, Av. Pádua Dias 11 CP 9, Piracicaba-SP 13418-900, Brazil
[b] Illinois Natural History Survey, Prairie Research Institute, University of Illinois, 1816 S. Oak St., Champaign, IL 61820, USA

HIGHLIGHTS

- *Cotesia flavipes* is used in >3 million ha/year against the sugarcane borer in Brazil.
- The effects of the pathogen *Nosema* sp. was assessed on *C. flavipes*.
- Heavily infected sugarcane borer larvae did not support parasitism.
- Life cycle was altered in parasitoids developing in infected sugarcane borers.
- *Nosema* sp. reduced the ability of the parasitoid to select and locate hosts.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 26 March 2012
Accepted 22 June 2012
Available online 1 July 2012

Keywords:
Nosematidae
Cotesia flavipes
Diatraea saccharalis
Insect pathology
Transmission
Biological control

ABSTRACT

A major issue for mass rearing of insects concerns sanitary conditions and disease. Microsporidian infection (*Nosema* sp.) in laboratory colonies of *Diatraea saccharalis* (Fabr.) (Lepidoptera: Crambidae), used in producing the parasitoid, *Cotesia flavipes* Cameron (Hymenoptera: Braconidae), is representative of the problems faced by growers and industry. Although *C. flavipes* has been produced for several years in Brazil for biological control of *D. saccharalis*, we have only recently observed that the parasitoid becomes infected when developing inside hosts infected with *Nosema* sp. We assessed the effects of *Nosema* sp. on *C. flavipes*, including the ability to locate and select hosts, and evaluated pathogen transmission. Third instar larvae of *D. saccharalis* were inoculated with *Nosema* sp. spores at different concentrations and were parasitized when larvae reached fifth instar. Heavily infected *D. saccharalis* larvae did not support parasitism. Parasitoids that developed in infected *D. saccharalis* larvae exhibited increased duration of larval and pupal stages, decreased adult longevity and number of offspring, and reduced tibia size compared to parasitoids developing in uninfected *D. saccharalis* larvae. Infection by *Nosema* sp. reduced the ability of the *C. flavipes* parasitoid to distinguish between volatiles released by the sugarcane infested by healthy larvae and pure air. Uninfected parasitoids preferred plants infested with uninfected hosts. But infected *C. flavipes* did not differentiate between uninfected hosts and those infected with *Nosema* sp. The pathogen is transmitted from host to parasitoids and parasitoids to hosts. Pathogenic effects of the microsporidium in *C. flavipes* are sufficiently severe to justify disease management efforts, particularly considering the importance of *C. flavipes* as a biological control agent in sugarcane.

* Corresponding author. Fax: +55 19 3429 4120.
E-mail address: delalibera@usp.br (I. Delalibera Jr.).

1049-9644
http://dx.doi.org/10.1016/j.biocontrol.2012.06.012

1. Introduction

Sugarcane is one of the most important agribusiness sectors for the Brazilian economy, comprising approximately 8 million cultivated hectares country-wide (União dos Produtores de Bioenergia (UPON), 2012). Herbivorous pests are a limiting factor in sugarcane production. The sugarcane borer, *Diatraea saccharalis* (Fabr.) (Lepidoptera: Crambidae), occurs from the southern region of the United States, through the Caribbean and Central America to Argentina (Capinera, 2001), and is considered the most important sugarcane pest in the western hemisphere (Long and Hensley, 1972). It is responsible for direct damage by stem boring and indirect damage by favoring penetration of opportunistic fungi (Gallo et al., 2002) into the plants. These fungi severely affect the yield by inciting stalk rot of sugarcane (Rago and Tokeshi, 2005). Sugar loss may also occur before fermentation when fungi-infected and healthy canes are crushed together, resulting in losses in both sugar and alcohol production. The parasitoid *Cotesia flavipes* Cameron (Hymenoptera: Braconidae), a gregarious larval endoparasitoid and cenobiont, was imported from Trinidad to Brazil in 1974 (Mendonça et al., 1977) as a biological control agent of *D. saccharalis*.

C. flavipes is inundatively released in more than three million hectares of sugarcane fields each year (http://www.faes.org.br). Females oviposit into the hemocoel of larvae and manipulate host physiology with venom and polydnavirus (Rodriguez-Perez and Beckage, 2008) to reduce immune response by the host and facilitate larval development (Scaglia et al., 2005). An average of 45 offspring per host (Campos-Farinha and Chaud-Netto, 2000) are produced. These parasitoids significantly impact population growth of the borers in sugarcane fields (Botelho and Macedo, 2002). To meet demand for control of the pest, the parasitoids are mass-produced in *D. saccharalis* larvae under laboratory conditions.

The primary challenges for mass rearing of *C. flavipes* in the laboratory involve sanitary problems, including pathogens. Occurrences of *Nosema* sp. (Microsporidia: Nosematidae) in laboratory populations of *D. saccharalis* have been reported in Brazil since the seventies (S.B. Alves, personal communication). Recently, we observed that prevalence of *Nosema* sp. is high in many laboratories producing *C. flavipes* in São Paulo State, Brazil.

Associations between parasitoids and the microsporidia infecting their hosts have been studied for several host-parasitoid systems. Brooks (1973) reviewed parasitoids that are susceptible to microsporidian pathogens of their hosts. Adverse effects observed in parasitoids that developed in hosts infected with microsporidia usually result from direct infection by the pathogen or from accumulation in the midgut of ingested spores (Andreadis, 1980). Andreadis (1980, 1982) described the effects of the pathogen *Nosema pyrausta* on the parasitoid *Macrocentrus grandii* Goidanich (Hymenoptera: Braconidae) of the European corn borer, *Ostrinia nubilalis* (Hübner) (Lepidoptera: Pyralidae), including prolonged pupal development and reduced longevity of *M. grandii*. Also, the percentage of *M. grandii* parasitism in field populations of *O. nubilalis* is inversely correlated with the prevalence of *N. pyrausta*. Siegel et al. (1986) evaluated the impact of *N. pyrausta* on *M. grandii* in Illinois, demonstrating that the infection levels of the pathogen in *O. nubilalis* corresponds with the levels found in the braconid. The authors also concluded that infected parasitoid females transmit *N. pyrausta* to their offspring and is a vector of the pathogen in laboratory rearing.

Studies of interactions among microsporidia and Braconidae revealed that the type and intensity of effects on the parasitoids reared in infected hosts are variable and depend on the pathogen and host species involved, generally including effects on development time, adult longevity, fecundity and oviposition of the host, as well as on parasitoid fitness, although in some cases such effects are not significant (Brooks and Cranford, 1972; Moawed et al., 1997; Hoch et al., 2000; Down et al., 2005).

Although *C. flavipes* is reared on a large scale to control *D. saccharalis* in Brazil, no studies have been conducted to determine the impact of *Nosema* sp. on parasitoids produced and released in field, information that is relevant to successful biological control programs in sugarcane plantations. Our study therefore aimed to assess the impact of *Nosema* sp. on *C. flavipes* development and host selection, and to elucidate pathogen transmission patterns under laboratory conditions.

2. Materials and methods

2.1. Preparing microsporidium inoculum

Nosema sp. spore suspensions were obtained by macerating infected *D. saccharalis* larvae in saline (NaCl 0.85%) +0.05% Pentabiótico Veterinário Reforçado (Fort Dodge® Saúde Animal Ltda comprised of Streptomycin, Benzocaine, Procaine and Penicillin). The spore suspensions were filtered through cheesecloth, centrifuged to remove host material and resuspended in saline solution (Undeen and Vávra, 1997). Spore concentration was determined using a Neubauer chamber.

2.2. Experimental infections of sugarcane borer

D. saccharalis larvae were reared on artificial diet (King and Hartley, 1985) to third instar. Before inoculation, the larvae were starved for 8 h. Inoculation of *D. saccharalis* larvae was carried out by transferring 5 μl spore suspension of 10^4, 10^5, 10^6, 10^7, and 10^8 spores/ml in saline per 100 mm^3 artificial diet disk prepared without Nipagin® (Metilparaben) and formaldehyde (representing an average of 10, 10^2, 10^3, 10^4 and 10^5 spores/larva, respectively). Disks were placed in disposable 60 × 15 mm Petri dishes, three disks per dish, and then 15 larvae were transferred to each dish. After the larvae consumed the entire diet portion, they were transferred into glass tubes (5 larvae/tube), containing approximately 15 ml of artificial diet and maintained at 26 ± 0.5 °C, 12:12 h photoperiod for 15 days. Ten fifth-instar larvae with similar head capsule size were selected from each Petri dish for use in the bioassays. The remaining individuals were analyzed microscopically to confirm infection.

2.3. Rearing uninfected and Nosema-infected C. flavipes

C. flavipes parasitoids were reared in infected and uninfected adult *D. saccharalis* obtained from the population of the Laboratory of Pathology and Microbial Control of Insects (ESALQ/USP). Fifth instar larvae of uninfected *D. saccharalis* and those infected by different concentrations (10, 10^2, 10^3, 10^4 and 10^5 spores/larva) of *Nosema* sp. were offered to the parasitoids for oviposition. Each larva was offered to only one *C. flavipes* female, allowing a single oviposition. The parasitoid females used to infect hosts were collected in Eppendorf tubes and examined for presence of microsporidian spores under phase contrast microscopy (1000× magnification) by smearing individual insects on a microscope slide in one drop of saline solution (0.85% NaCl). Parasitized fifth instar *D. saccharalis* larvae were maintained in disposable Petri dishes (60 × 15 mm) containing artificial diet (King and Hartley, 1985), at 26 ± 1 °C and 12:12 h photoperiod. After parasitoid pupation, the infected and uninfected *D. saccharalis* hosts were evaluated under phase contrast microscopy to confirm presence or absence of *Nosema* sp. spores. After emergence, *C. flavipes* females were placed with the males

for 24 h to allow mating before being used in bioassays. They were reared in growth chambers at 26 ± 1 °C, 75% ± 10% relative humidity, 12:12 h photoperiod.

2.4. Effects of Nosema sp. on C. flavipes development

Six treatments included uninfected *D. saccharalis* larvae fed diet inoculated with sterile saline or infected larvae fed diet inoculated with *Nosema* sp. spores at concentrations (10, 10^2, 10^3, 10^4 and 10^5 spores/larva). Each treatment consisted of four replications with ten fifth instar larvae each. Uninfected and infected *D. saccharalis* larvae were individually offered to single uninfected *C. flavipes* females, allowing one oviposition per larva. Each parasitized larva was transferred to disposable 60 × 15 mm Petri dishes containing a diet disc (883 mm^3) for maintenance after parasitism.

Parasitized uninfected and infected larvae were maintained at 26 ± 0.5 °C, 12:12 h photoperiod until the parasitoids reached pupal stage. Host larvae were observed daily and diet was changed every 5 days; dead larvae and pupae were removed. *C. flavipes* is gregarious with multiple cocoons forming a single mass per host larva. Cocoons from each uninfected and infected larva host were transferred to individual clean disposable 60 × 15 mm Petri dishes and held under the same environmental conditions previously detailed. The number of dead parasitoid larvae was determined during this step.

The number of *C. flavipes* females, males, and inviable pupae were quantified soon after adult *C. flavipes* emerged from the pupa. The following biological parameters were determined for *C. flavipes*: duration of larval and pupal periods, number of pupae and adults, longevity, size of female and male tibias, and percentage of *C. flavipes* successfully emerging from uninfected *D. saccharalis* larvae and those infected by *Nosema* sp. at different concentrations of inoculum.

To assess the effect of *Nosema* sp. on the second generation of *C. flavipes*, the adults reared in uninfected host larvae and those reared in infected larvae in the previous experiment were used to parasitize uninfected *D. saccharalis* larvae. For this experiment, 10 female *C. flavipes* from each treatment were randomly selected and used to parasitize uninfected fifth instar *D. saccharalis* larvae, one female parasitoid/larva. After oviposition, the remaining parasitoids from each treatment were evaluated for longevity, size (measuring tibia length of the last pair of legs), and presence of *Nosema* sp. spores. Parasitized *D. saccharalis* larvae were maintained in growth chambers under the previously described conditions until the second generation of *C. flavipes* emerged. *D. saccharalis* larvae were examined for presence of *Nosema* sp. after parasitoid pupation. At emergence, second generation *C. flavipes* were evaluated for percent emergence, duration of larval and pupal periods, number of pupae and adults, and longevity. The *C. flavipes* adults obtained during this step were also examined for presence of pathogen spores in their tissues.

2.5. Effects of Nosema sp. on host location and selection by C. flavipes

Preliminary tests determined that *C. flavipes* females were attracted to 20-cm tall sugarcane plants (variety SP-801842) injured by fourth instar *D. saccharalis* larvae, together with their feces. Parasitoids responded to the plants in $\leqslant$5 min. Treatment sets were composed of the host plant plus uninfected larvae or *Nosema*-infected larvae and their associated fresh feces. Groups of five newly emerged *D. saccharalis* larvae were placed into glass tubes containing artificial diet for 10 days. When larvae reached third instar they were transferred to 60 mm diameter Petri dishes and inoculated experimentally with 10^5 spores/larva (as previously described); control larvae were fed diet with sterile saline. The larvae were held in the Petri dishes for 3 days, and then five larvae were transferred to each sugarcane plant and held for 24 h to allow larvae to bore into the plant stem before the bioassays were performed.

We used a glass Y-olfactometer, 27 mm diameter, 14 cm length (arm), with airflow of 0.4 L/minute to assay *C. flavipes* response to the host on plant material. The respective treatments were placed at the end of the olfactometer arms; a single parasitoid was introduced into the entrance of the common stem and observed for up to 5 min. Three bioassays were performed using healthy parasitoids and two bioassays with infected parasitoids. In each bioassay, two treatments were compared at a time. Healthy parasitoids were tested as follows: (i) plant + uninfected *D. saccharalis* larvae vs. pure air; (ii) plant + uninfected *D. saccharalis* larvae vs. plant material; and (iii) plant + uninfected *D. saccharalis* larvae vs. plant + *Nosema*-infected *D. saccharalis* larvae. Parasitoids infected by *Nosema* sp. were tested in the following bioassays: (iv) plant + uninfected *D. saccharalis* larvae vs. pure air; and (v) plant + uninfected *D. saccharalis* larvae vs. plant + *Nosema*-infected *D. saccharalis* larvae.

After each test, the parasitoids were microscopically examined to confirm presence or absence of *Nosema* sp. spores. In addition, infected and uninfected *D. saccharalis* larvae and their feces were examined for presence of spores. Each bioassay was performed over a 4-day period, and at least 10 parasitoids were tested per day, totaling 40 females per bioassay, except bioassay 3 where 60 females were used. Statistical analyses were performed using the R–2.14.0 program (Windows). The differences between the behavioral responses of the treatment pairs were analyzed by *chi-square* test ($\alpha = 0.01$ and 0.05). Individuals that did not make a choice were excluded from statistical analysis.

2.6. Transmission of Nosema sp. in C. flavipes

In earlier experiments, we determined that *Nosema*-infected *C. flavipes* could infect *D. saccharalis* during oviposition. We selected infected parasitoids from each of four subsequent generations to parasitize uninfected *D. saccharalis* to determine the transmission pattern.

Newly emerged *Nosema*-infected *C. flavipes* females were held with males for 24 h for mating. Each *C. flavipes* female was used to parasitize a single uninfected fifth instar *D. saccharalis* larva, allowing a single oviposition. *C. flavipes* used in this step constituted the F_0 generation of the bioassay. Next, each larva was transferred to a disposable 60 × 15 mm Petri dish containing a 883 mm^3 artificial diet disc to maintain the larvae after *C. flavipes* parasitism, maintained in a growth chamber at 26 ± 0.5 °C 12 h photoperiod, until the parasitoid pupal phase. Larvae were observed daily and the diets were changed every 5 days, removing dead and pupated *D. saccharalis* during this period. Each *C. flavipes* cocoon mass was transferred to a clean disposable 60 × 15 mm Petri dish and maintained under the previously described conditions. Infected *C. flavipes* adults from the F_1 generation were maintained in growth chambers for 24 h and, after this period, approximately 10 females from each cocoon mass were selected and used to parasitize uninfected fifth instar *D. saccharalis* larvae, one oviposition per host, resulting in an F_2 generation of parasitoids. Successive parasitizations were made until obtaining F_4 parasitoids.

After parasitism, all *C. flavipes* females used to parasitize hosts in the bioassays were individually examined to confirm the presence of *Nosema* sp. spores and estimate the level of infection for each. For the control treatment, the same procedure was conducted using uninfected *C. flavipes* to parasitize uninfected *D. saccharalis* larvae.

C. flavipes adults from each generation were randomly selected and macerated in 200 μl of saline using a micropestle and microscopically examined for presence of *Nosema* spores. *D. saccharalis* larvae were examined after parasitoid pupation by removing small

midgut tissue samples for analysis. The level of infection in the individual parasitoids and host larvae was also estimated by using a pre-established scale for number of spores per microscope field: (+) for 1–5, (++) for 6–15, and (+++) for $\geqslant$16 spores/microscopic field.

We performed a regression analysis (ANOVA) to analyze the relationship between the percentage of infected progeny of *C. flavipes* (variable "*x*") and the percentage of infected hosts, *D. saccharalis* (variable "*y*").

3. Results

3.1. Effects of Nosema sp. on C. flavipes development

When *C. flavipes* developed in *D. saccharalis* larvae inoculated with an average of 100 *Nosema* sp. spores/larva, 76.9% ± 0.2% of the parasitoids became infected in the first generation (F_1), significantly higher infection levels than observed for lower dosages ($F_{5,16}$ = 74,69; $P < 0.0001$) (Table 1). Of the parasitoids that developed in larvae ingesting more than 100 spores, 100% became infected. The percent infection in F_2 generation *C. flavipes*, obtained from oviposition by the F_1 in uninfected host larvae, was lower than in F_1 adults. Thirty-three percent was the highest percentage of infected parasitoids in F_2, differing only from those observed in *C. flavipes* developed in *D. saccharalis* larvae inoculated with 0 and 10 *Nosema* sp. spores/larva ($F_{4,15}$ = 4.25; P = 0.0169) (Table 1). Presence of pathogen spores in tissues of F_1 and F_2 adults indicate that this *Nosema* sp. was transferred between stages of parasitoid development.

Early mortality of *D. saccharalis* larvae inoculated at average dosages higher than 10^4 spores per individual and parasitized by *C. flavipes* was $\geqslant$75% ($F_{5,18}$ = 64,68; P < 0,0001), preventing most of the parasitoids from completing their life cycle (Table 1). Host mortality and, consequently, parasitoid mortality was so high for treatments of 10^5 spores/larva that no viable *C. flavipes* females were obtained; therefore, it was not possible to assess the F_2 generation in this treatment.

Parasitoids that developed in *D. saccharalis* larvae inoculated with $\geqslant 10^3$ *Nosema* sp. spores were negatively affected in all tested biological parameters, indicating that *Nosema* sp. is pathogenic to *C. flavipes* (Table 2). Duration of parasitoid larval stage ($F_{4,20}$ = 10.81; $P < 0.0001$) and pupal stage ($F_{4,118}$ = 11.08; $P < 0.0001$) increased and adult longevity ($F_{4,118}$ = 16,14; P < 0,0001) was reduced relative to increasing dosage in the hosts. The number of *C. flavipes* pupae ($F_{4,120}$ = 20.51; $P < 0.0001$) and adults ($F_{4,118}$ = 21.35; $P < 0.0001$) was reduced by half in host larvae fed 10^4 spores compared to those developing in uninfected larvae. *C. flavipes* adults obtained from heavily infected larvae exhibited lower emergence percentage ($F_{4,120}$ = 7.97; $P < 0.0001$) and tibia size was smaller for both females ($F_{4,88}$ = 14.46; $P < 0.0001$) and males ($F_{4,86}$ = 8.13; $P <$ 0.0001) than for adults produced in uninfected larvae or those with low-level infections.

The effects of *Nosema* sp. on the F_2 generation of *C. flavipes* originating from F_1 females that developed in infected hosts were less severe than those observed in the F_1 generation. There were statistically significant differences in duration of the larval stage ($F_{4,88}$ = 6.43; $P < 0.0001$) and adult stage ($F_{4,88}$ = 6.92; $P < 0.0001$) and in percent emergence ($F_{4,88}$ = 1.83; P = 0.1298) comparing the treatment that received 10^4 spores/larva with the control treatment (Table 3). The larval stage trended longer, and reduced emergence was evident at lower dosages, but were not significantly different from uninfected controls. It was not possible to include in the statistical analysis the results for the treatment with an average dosage of 10^5 spores/larva, due to the high mortality in this treatment (only one and two *D. saccharalis* larvae survived in the F_1 and F_2 generations, respectively).

3.2. Effects of Nosema sp. on host location and selection by C. flavipes

Response of parasitoids to olfactory stimuli "plant + uninfected larva" was significantly higher than response to pure air (DF = 1, x^2 = 12.1, P = 0.0005). We also observed that *C. flavipes* females were more attracted to plants with herbivory than plants without (DF = 1, x^2 = 22.5, P = 0.000002). Uninfected *C. flavipes* females were more attracted to the plants with herbivory by uninfected host larvae, compared to plants with infected larvae (DF = 1, x^2 = 11.3, P = 0.0008) (Fig. 1).

Nosema-infected *C. flavipes* females did not distinguish between the olfactory stimuli released in herbivory by uninfected hosts vs. hosts with nosemosis (DF = 1, x^2 = 3.6, P = 0.06), but distinguished between volatiles released in herbivory by uninfected hosts vs. pure air (DF = 1, x^2 = 4.9, P = 0.03) (Fig. 2). High levels of *Nosema* infection apparently reduced the ability of female wasps to select a *D. saccharalis* host.

The average response time of *C. flavipes* females to olfactory stimuli in the Y-olfactometer was 2.44 and 2.66 min for uninfected and infected parasitoids, respectively. The average percentage of insects that did not respond to the volatiles tested were 35.1% and 44.4% for uninfected and infected parasitoids, respectively.

3.3. Transmission of Nosema sp. in C. flavipes

On average, 78% of the pupal masses obtained in the four trials of *C. flavipes* were infected by the pathogen (Fig. 3). The number of infected progeny, independent of infection level, decreased in the first three trials (F_1, F_2, and F_3) from 96.3% to 50%, increasing in the fourth trial to 89.5%.

On average, 70% of *D. saccharalis* larvae parasitized by *Nosema*-infected *C. flavipes* females became infected, indicating that this species is capable of efficiently transmitting the pathogen to its hosts. Host infection rates varied between 62.8% and 75.6% in the four trials. *Nosema* sp. spores were not found in *D. saccharalis* larvae maintained as controls in the bioassay.

There was a significant linear relationship (P = 0.00056) dependency between the percentage of progenies of infected *C. flavipes* and the percentage of infected hosts, *D. saccharalis*, according to the model: $y = 0.46 + 13.36 \times$ (F = 19.73, R^2 = 0.58).

4. Discussion

Infection by *Nosema* sp. in *D. saccharalis* not only impairs *C. flavipes* production due to host mortality before the parasitoid completes its cycle, but also affects the life cycle and behavior of the parasitoid. Increased early mortality in parasitized larvae is directly proportional to increased dosage, and high dosages produce heavy infections that prevent the parasitoid from completing its development cycle. Similarly, Hoch et al. (2000) reported that *Vairimorpha* sp. infection in *Lymantria dispar* (L.) (Lepidoptera: Lymantriidae) concomitant with parasitism by *Glyptapanteles liparidis* (Bouché) (Hymenoptera: Braconidae) results in significantly increased early mortality in *L. dispar* compared to mortality observed only due to the microsporidia.

Microsporidian infections in parasitoids and their hosts are not uncommon (Becnel and Andreadis, 1999). Contamination usually occurs when the parasitoid larvae feed on hemolymph and tissues of infected hosts and can result in a variety of deleterious effects on the parasitoids. Futerman et al. (2005) discussed that effects of microsporidia on the biology of parasitoids; hymenoptera parasitoids in most cases are susceptible to microsporidia pathogenic to

Table 1
Average percentage of *Cotesia flavipes* adults acquiring *Nosema* sp. while developing in *Diatraea saccharalis* larvae exposed to different pathogen dosages (first generation), and in the second generation, after F_1 adults parasitized uninfected larvae.

Dosage (spores/*D. saccharalis* larva)	C. flavipes[a] F_1 generation		C. flavipes[b] F_2 generation
	C. flavipes with Nosema sp. (%) ± SE	Mortality of *D. saccharalis* after parasitism (%) ± SE*	*C. flavipes* infected with *Nosema* sp. (%) ± SE
0	0 ± 0 a	0 ± 0 a	0 ± 0 a
1×10^1	7.1 ± 0.1 a	0 ± 0 a	0 ± 0 a
1×10^2	76.9 ± 0.2 b	12.2 ± 0.1 a	7.1 ± 0.1 ab
1×10^3	100.0 ± 0 b	20.0 ± 0.1 a	18.3 ± 0.1 ab
1×10^4	100.0 ± 0 b	75.0 ± 0.1 b	33.3 ± 0.2 b
1×10^5	100.0 ± 0 b	92.5 ± 0.1 b	–

Averages in the columns followed by the same letter do not differ statistically by Tukey test ($\alpha = 0.05$).
**Mortality prior to maturation of *C. flavipes* in the host.
[a] First generation *C. flavipes* developed in *D. saccharalis* infected by *Nosema* sp. at different concentrations.
[b] Second generation *C. flavipes*, progeny of infected parasitoids that developed in initially uninfected larvae.

Table 2
Effects of microsporidian infection in *Diatraea saccharalis* on F_1 generation *Cotesia flavipes* developing in larvae inoculated with different dosages of *Nosema* sp. spores.

Dosage (spores/*D. saccharalis* larva)	*C. flavipes* developing in *D. saccharalis* infected with *Nosema* sp. at different concentrations							
	Duration of life stages (Days ± SE)			Number (mean ± SE) of *C. flavipes*/*D. saccharalis* larva		Emergence (%) ± SE	Tibia size (mm) (mean ± SE)	
	Larva	Pupa	Adult	Pupae	Adults		Female	Male
0	11.1 ± 0.1 a	5.4 ± 0.1 a	3.6 ± 0.1 a	64.3 ± 3.9 a	62.0 ± 3.7 a	95.9 ± 0.03 a	0.61 ± 0.01 a	0.61 ± 0.01 a
1×10^1	11.7 ± 0.4 ab	6.2 ± 0.1 b	3.1 ± 0.1 a	66.6 ± 3.2 a	64.0 ± 3.7 a	94.8 ± 0.04 a	0.61 ± 0.01 a	0.62 ± 0.01 a
1×10^2	11.3 ± 0.2 ab	6.2 ± 0.1 b	3.1 ± 0.1 a	53.6 ± 2.9 a	50.4 ± 2.5 a	94.2 ± 0.03 ab	0.57 ± 0.01 ab	0.58 ± 0.01 ab
1×10^3	12.7 ± 0.3 b	6.1 ± 0.1 b	2.5 ± 0.1 b	32.7 ± 2.8 b	29.5 ± 2.9 b	73.8 ± 2.75 c	0.53 ± 0.01 bc	0.56 ± 0.01 b
1×10^4	14.0 ± 0.1 c	6.3 ± 0.3 b	2.2 ± 0.2 b	33.4 ± 6.2 b	30.2 ± 6.6 b	77.8 ± 0.1 bc	0.51 ± 0.02 c	0.55 ± 0.02 c

*Averages in the columns followed by the same letter do not statistically differ by Tukey test at 5% significance level.

Table 3
Effects of *Nosema* sp. on F_2 generation *Cotesia flavipes* developing in uninfected *Diatraea saccharalis* larvae; F_2 are progeny of parasitoids that developed in host larvae inoculated at different dosages of *Nosema* sp. spores.

Dosage (spores/*D. saccharalis* larva) in F_1 larvae of *C. flavipes*	F_2 of *C. flavipes* developing in healthy *D. saccharalis*					
	Average duration of life stages (days)			Average No. of *C. flavipes*/*D. saccharalis* larva		Emergence (%) ± SE
	Larval ± SE	Pupal ± SE	Adult ± SE	Pupae ± SE	Adults ± SE	
0	11.1 ± 0.2 a	5.6 ± 0.1 ab	3.1 ± 0.1 a	58.3 ± 4.8 a	57.6 ± 4.5 a	99.3 ± 0.01 a
1×10^1	12.2 ± 0.2 bc	6.1 ± 0.2 a	2.1 ± 0.1 c	60.6 ± 5.9 a	59.8 ± 5.9 a	98.6 ± 0.02 ab
1×10^2	12.1 ± 0.3 bc	6.0 ± 0.2 a	2.6 ± 0.3 abc	49.6 ± 5.8 a	48.5 ± 5.7 a	97.4 ± 0.03 ab
1×10^3	11.5 ± 0.1ab	5.8 ± 0.2 a	2.2 ± 0.1 bc	57.5 ± 3.7 a	56.6 ± 3.7 a	98.2 ± 0.02 ab
1×10^4	12.4 ± 0.4 c	5.0 ± 0.4 b	2.9 ± 0.3 ab	63.7 ± 8.4 a	61.9 ± 2.5 a	97.2 ± 0.02 b

*Averages in the columns followed by the same letter do not statistically differ by Tukey test at 5% significance level.

their hosts (Thomson, 1960). Nevertheless, some microsporidia species were reported to be specific to parasitoids, i.e., *Nosema muscidifuracis*, *Nosema cardiochilis*, and *Nosema campoletidis* (Brooks and Cranford, 1972; Geden et al., 1995).

In this study, we observed increased duration of immature parasitoid stages, similar to effects reported by Hoch et al. (2000) and Down et al. (2005), whereas Brooks and Cranford (1972) observed no delay in host development. Moawed et al. (1997), Geden et al. (2003), and Futerman et al. (2005) did not observe delayed development in the life cycles of parasitoids in the Families Braconidae, Encyrtidae, and Pteromalidae caused by microsporidia in the genera *Nosema* and *Tubulinosema*. *N. pyrausta* infections did not appear to adversely affect the ability of *M. grandii* larvae to pupate; however, adult emergence was reduced by more than 38% (Andreadis, 1980). Additionally, Andreadis (1980) reported that longevity of *N. pyrausta*-infected adult survivors of both sexes was shorter than that of uninfected controls.

Infection of *D. saccharalis* by *Nosema* sp. resulted in deleterious effects on emergence, fecundity, longevity, and size of *C. flavipes*. Similar effects were reported for other parasitoid species (Saleh et al., 1995; Moawed et al., 1997; Schuld et al., 1999; Hoch et al., 2000; Down et al., 2005; Futerman et al., 2005). These effects were less evident in the second generation of parasitoids. However, infected parasitoid larvae fed on uninfected hosts that may be more nutritionally favorable. Transmission of *Nosema* sp. may be either vertical from female parasitoid to offspring, or horizontal to the host by the parasitoid female (mechanical transmission) and then infection of feeding larvae once the host becomes infected.

Researchers initially attributed the negative impacts of Microsporidia on parasitoids to undigested spore masses accumulated in the digestive tract of parasitoid larvae, leading to nutritional imbalances (Thomson, 1958; Laigo and Tamashiro, 1967; Cossentine and Lewis, 1986, 1988; Moawed et al., 1997). Hoch et al. (2002) studied the nutritional quality of gypsy moth hosts infected

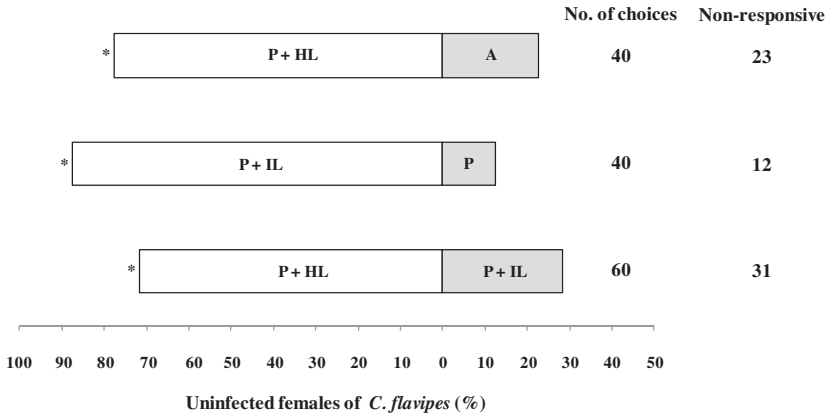


Fig. 1. Response of uninfected *Cotesia flavipes* females to volatiles released by sugarcane plants damaged by uninfected (healthy) fourth instar larvae of *Diatraea saccharalis* (*P* + HL): vs. pure air (A); vs. sugarcane plant without herbivory (*P*); or vs. sugarcane plant damaged by fourth instar larvae of *D. saccharalis* infected with *Nosema* sp. (*P* + IL). *Significant by *Chi-square* test ($\alpha = 0.01$).

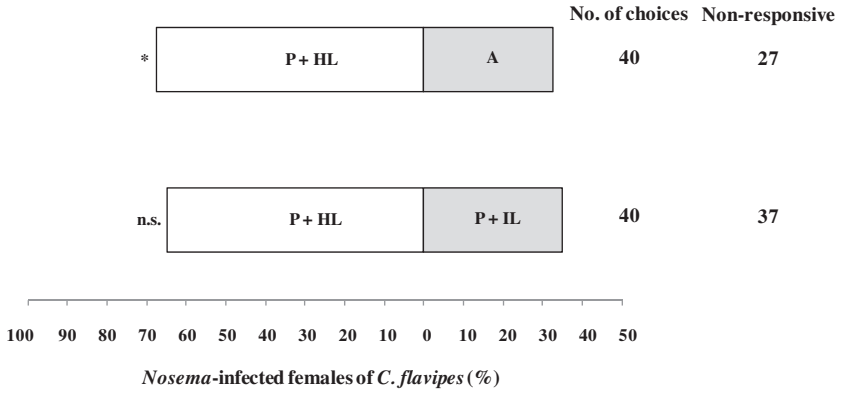


Fig. 2. Response of *Cotesia flavipes* females infected with *Nosema* sp. to volatiles released by sugarcane plants damaged by uninfected (healthy) fourth instar larvae of *Diatraea saccharalis* (*P* + HL) regarding: vs. pure air (A); or vs. sugarcane plant damaged by fourth instar larvae of *D. saccharalis* infected by *Nosema* sp. (*P* + IL).*Significant and n.s. not significant by *Chi-square* test (α=0.05).

with *Vairimorpha* sp. (*disparis*) and believe that changes in levels of carbohydrates and fatty acids in the host larvae are sufficiently severe to at least partially explain adverse effects on *G. liparidis* parasitoid development. Adult *M. grandii* parasitizing the European corn borer emerged from 90% of cocoons when uninfected but from only 14% of cocoons when infected with *N. pyrausta*. Most of the observed parasitoid mortality occurred during the fourth instar larval stage after exiting from the corn borer host (Siegel et al., 1986).

Infection by *Nosema* sp. reduced the ability of the *C. flavipes* parasitoid to distinguish between volatiles released by the sugarcane infested by healthy larvae and pure air. In corn borer populations where infections with *N. pyrausta* exceeded 45%, there was a highly significant inverse correlation between the prevalence of infection with *N. pyrausta* and the parasitism by *M. grandii*, with 88% of the variation in parasitism associated with the variation in infection prevalence (Andreadis, 1982). In addition, infections by *Nosema* sp. were described as being substantially able to limit the parasitoid's ability to survive for a sufficient length of time to locate and parasitize hosts in the field (Futerman et al., 2005). Geden et al. (1992) also demonstrated that *Muscidifurax raptor* Girault & Sanders (Hymenoptera: Pteromalidae) parasitoids infected by *N. muscidifuracis* have reduced reproductive potential, longevity, and ability to locate and parasitize their hosts.

Uninfected parasitoids preferred plants infested with uninfected hosts. In contrast, infected parasitoids did not differentiate between uninfected hosts and those infected with *Nosema* sp. The preference of uninfected parasitoids for plants infested by uninfected *D. saccharalis* larvae may be due to volatile emission induced by the higher activity of uninfected larvae. Although it was not quantified, we observed that nosemosis significantly reduced larval feeding, resulting in less damage inflicted to the plant and production of feces. Potting et al. (1995) suggest that the major source of volatiles in a plant-host complex is the injured plant itself, and the feces produced by the herbivores. In contrast, studies with the braconid, *G. liparidis*, concluded that there was no difference in parasitism percentage between non-infected larvae of *L. dispar* and those infected by *Vairimorpha* sp. (Hoch et al., 2000), and *Trichogramma nubilale* Ertle & Davis (Hymenoptera: Trichogrammatidae) do not discriminate between non-infected eggs and those infected by *N. pyrausta* (Saleh et al., 1995).

Nosema sp. transmission to the progeny of *C. flavipes* that developed in infected *D. saccharalis*, independent of inoculum dosage ingested by larvae, suggests that consumption of infected host tissues is an important transmission route. According to Futerman et al. (2005), there are several possible explanations for different levels of impact experienced by the parasitoids, among them, the fact that the parasitoids can effectively ingest a larger dose of spores than their host consumes, considering that the pathogen can be present at high densities in the host's tissues when infections are fully developed. Vertical transmission of Microsporidia in insects is very common (Geden et al., 1995; Becnel and Andreadis, 1999; Wittner and Weiss, 1999), however, the transmission patterns in parasitoids are variable. Transmission ranges

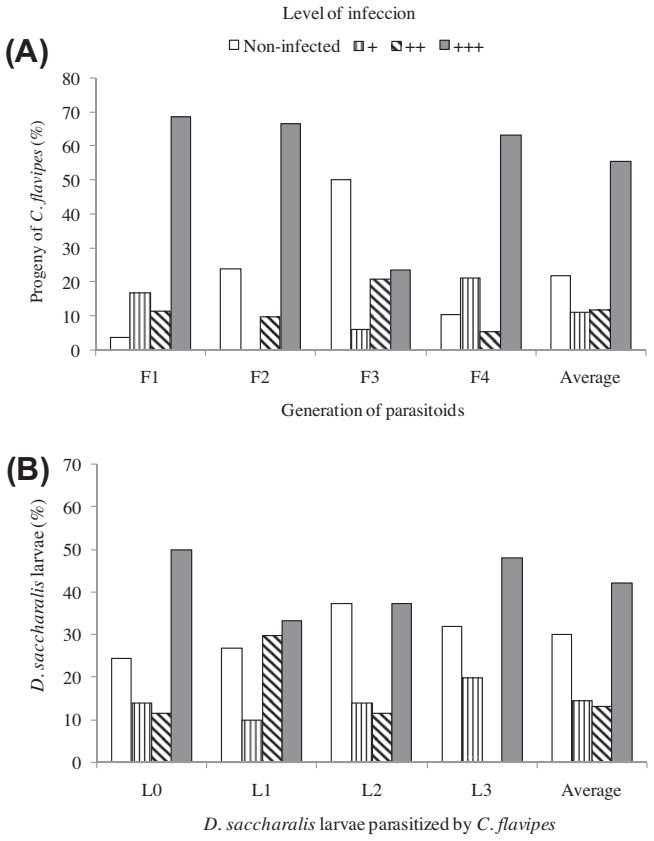


Fig. 3. Transmission of *Nosema* sp. between generations of *Cotesia flavipes* and their *Diatraea saccharalis* host. (A) Percentage of infected and non-infected *C. flavipes* progeny at different levels during four generations from F_0 parasitoids with nosemosis. (B) Percentage of *D. saccharalis* larvae that were infected and the level of infection after being parasitized by *C. flavipes* with nosemosis (L_0 larvae were used for producing parasitoids F_1, L_1 for F_2, and so on successively). Level of infeccion: (+) for 1–5, (++) for 6–15, and (+++) for $\geqslant$16 *Nosema* sp. spores/microscopic field.

from very efficient in some species (Brooks and Cranford, 1972; Geden et al., 1995) to non-existent in others (Hoch et al., 2000; Futerman et al., 2005). Our study has shown that *Nosema* sp. is transmitted through four generations of *C. flavipes*; however, we do not yet know whether this is the result of vertical transmission or horizontal transmission from parasitoid to host to parasitoid offspring.

The effects of the *Nosema* sp. pathogen on the parasitoid *C. flavipes* under laboratory conditions are sufficiently severe to justify disease mitigation efforts for mass rearing facilities, considering the importance of *C. flavipes* as a biological control agent of *D. saccharalis* in sugarcane.

Acknowledgments

The first and second authors are recipients of scholarships from the National Council for Scientific and Technological Development (CNPq).

References

Andreadis, T.G., 1980. *Nosema pyrauta* infection in *Macrocentrus grandii*, a braconid parasite of the European corn borer, *Ostrinia nubilalis*. J. Invertebr. Pathol. 35, 229–233.

Andreadis, T.G., 1982. Impact of *Nosema pyrauta* on field populations of *Macrocentrus grandii*, an introduced parasite of the European corn borer, *Ostrinia nubilalis*. J. Invertebr. Pathol. 39, 298–302.

Becnel, J.J., Andreadis, T.G., 1999. Microsporidia in Insects. In: Wittner, M., Weiss, L.M. (Eds.), The Microsporidia and Microsporidiosis. ASM Press, Washington DC, pp. 447–501.

Botelho, P.S.M., Macedo, N., 2002. *Cotesia flavipes* para o controle de *Diatraea saccharalis* (*Cotesia flavipes* for controlling *Diatraea saccharalis*). In: Parra, J.R.P., Botelho, P.S.M., Corrêa-Ferreira, B.S., Bento, J.M.S. (Eds.), Controle biológico no Brasil: parasitóides e predadores (Biological control in Brazil: parasitoids and predadors). Manole, São Paulo, pp. 477–494.

Brooks, W.M., 1973. Protozoa: host-parasite-pathogen interrelationships. Misc. Pub. Entomol. Soc. Am. 9, 105–111.

Brooks, W.M., Cranford, J.D., 1972. Microsporidioses of the hymenopterous parasites, *Campoletis sonorensis* and *Cardiochiles nigriceps*, larval parasites of *Heliothis* species. J. Invertebr. Pathol. 20, 77–94.

Campos-Farinha, A.E. de C., Chaud-Netto, J., 2000. Biologia reprodutiva de *Cotesia flavipes* (Cameron) (Hymenoptera: Braconidae) V. Avaliação do número de posturas, prole e razão sexual do parasitóide em relação ao tamanho do hospedeiro *Diatraea saccharalis* Fabricius (Lepidoptera: Pyralidae). Arq. Inst. Biol., São Paulo, 67 (2): pp. 249–252.

Capinera, J.L., 2001. Sugarcane Borer, *Diatraea saccharalis* (Fabricius) (Insecta:Lepidoptera: Pyralidae). Featured Creatures. Department of Entomology and Nematology, Division of Plant Industry, Extension Digital Information Source. <http://creatures.ifas.ufl.edu/field/sugarcane_borer.htm, 1-5>.

Cossentine, J.E., Lewis, L.C., 1986. Impact of *Vairimorpha necatrix* and *Vairimorpha* sp. (Microspora: Microsporidia) on *Bonetia comta* (Diptera: Tachinidae) within *Agrotis ipsilon* (Lepidoptera: Noctuidae) hosts. J. Invertebr. Pathol. 47, 303–309.

Cossentine, J.E., Lewis, L.C., 1988. Impact of *Nosema pyrausta*, *Nosema* sp., and a nuclear polyhedrosis virus on *Lydella thompsoni* within infected *Ostrinia nubilalis* hosts. J. Invertebr. Pathol. 51, 126–132.

Down, R.E., Smethurst, F., Bell, H.A., Edwards, J.P., 2005. Interactions between the solitary endoparasitoid, *Meteorus gyrator* (Hymenoptera: Braconidae) and its host, *Lacanobia oleracea* (Lepidoptera: Noctuidae), infected with the entomopathogenic microsporidium, *Vairimorpha necatrix* (Microspora: Microsporidia). B. Entomol. Res. 95 (2), 133–144.

Futerman, P.H., Layen, S.J., Kotzen, M.L., Franzen, C., Kraaijeveld, A.R., 2005. Fitness effects and transmission routes of a microsporidian parasite infecting *Drosophila* and its parasitoids. Parasitology 132, 479–492.

Gallo, D., Nakano, O., Silveira Neto, S., Carvalho, R.P.L., Batista, G.C. de, Berti Filho, E., Parra, J.R.P., Zucchi, R.A., Alves, S.B., 2002. Pragas das plantas e seu controle (Pests in plants and their control). In: Gallo, D., Nakano, O., Silveira Neto, S., Carvalho, R.P.L., Batista, G.C. de, Berti Filho, E., Parra, J.R.P., Zucchi, R.A., Alves, S.B. (Eds.), Manual de entomologia agrícola (Manual of agricultural entomology). Agronômica Ceres, Piracicaba, pp. 397–912.

Geden, C.J., Almeida, M.A.F. de, Prado, A.P. do., 2003. Effects of *Nosema* disease on fitness of the parasitoid *Tachinaephagus zealandicus* (Hymenoptera: Encyrtidae). Biol. Control. 32 (5), 1139–1145.

Geden, C.J., Long, S.J., Rutz, D.A., Becnel, J.J., 1995. *Nosema* disease of the parasitoid *Muscidifurax raptor* (Hymenoptera: Pteromalidae): prevalence, patterns of transmission, management, and impact. Biol. Control. 5, 607–614.

Geden, C.J., Smith, L., Long, S.J., Rutz, D.A., 1992. Rapid deterioration of searching behavior, host destruction, and fecundity of *Muscidifurax raptor* (Hymenoptera: Pteromalidae) in culture. Entomol. Soc. Am. 85 (2), 179–187.

Hoch, G., Schafellner, C., Henn, M.W., Schopf, A., 2002. Alterations in carbohydrate and fatty acid levels of *Lymantria dispar* larvae caused by a microsporidian infection and potential adverse effects on a co-occurring endoparasitoid *Glyptapanteles liparidis*. Arch. Insect Biochem. Physiol. 50, 109–120.

Hoch, G., Schopf, A., Maddox, J.V., 2000. Interactions between an entomopathogenic microsporidium and the endoparasitoid *Glyptapanteles liparidis* within their host, the gypsy moth larva. J. Invertebr. Pathol. 75, 59–68.

King, E.G., Hartley, G.G., 1985. *Diatraea saccharalis*, in: Singh, R. F. (Ed.), Moore Handbook of insect rearing, vol. 2, Elsevier, Amsterdam, pp. 265-270.

Laigo, F.M., Tamashiro, M., 1967. Interactions between a microsporidian pathogen of the lawn-armyworm and the hymenopterous parasite *Apanteles marginiventris*. J. Invertebr. Pathol. 9, 546–554.

Long, W.H., Hensley, S.D., 1972. Insect pests of sugar cane. Annu. Rev. Entomol. 17, 149–176.

Mendonça, A.F., Risco, S.H., Costa, J.M.B., 1977. Introduction and rearing of *Apanteles flavipes* Cameron (Hymenoptera: Braconidae) in Brazil. Proc. Int. Soc. Sugarcane Technol. 1, 703–710.

Moawed, S.M., Marei, S.S., Saleh, M.R., Matter, M.M., 1997. Impact of *Vairimorpha ephestiae* (Microspora: Nosematidae) on *Bracon hebetor* (Hymenoptera: Braconidae), an external parasite of the American bollworm, *Heliothis armigera* (Lepidoptera: Noctuidae). Eur. J. Entomol. 94, 561–565.

Potting, R.P.J., Vet, L.E.M., Dicke, M., 1995. Host microhabitat location by stemborer parasitoid *Cotesia flavipes*: the role of herbivore volatiles and locally and systemically induced plant volatiles. J. Chem. Ecol. 21, 525–539.

Rago, A., Tokeshi, H., 2005. Doenças da Cana-de-açúcar (Diseases of sugarcane). In: Kimati, H., Amorin, L., Bergamin Filho, A., Camargo, L.E.A., Rezende, J.A.M. (Eds.), Manual de fitopatologia: doenças das plantas cultivadas (Manual of plant pathology: diseases of cultivated plants), fourth ed. Vol. 2. Agronômica Ceres, São Paulo, pp. 185–196.

Rodriguez-Perez, M.A., Beckage, N.A., 2008. Comparison of three methods of parasitoid polydnavirus genomic DNA isolation to facilitate polydnavirus genomic sequencing. Arch. Insect Biochem. 67, 202–209.

Saleh, M.M.E., Lewis, L.C., Obrycki, J.J., 1995. Selection of *Nosema pyrausta* (Microsporidia: Nosematidae)-infected *Ostrinia nibilalis* (Lepidoptera: Pyralidae) eggs for parasitization by *Trichogramma nubilale* (Hymenoptera: Trichogrammatidae). Crop Protection 14 (4), 327–330.

Scaglia, M., Chaud-Netto, J., Brochetto-Braga, M.R., Ceregato, S.A., Gobbi, N., Rodrigues, A., 2005. Oviposition sequence and offspring of mated and virgin females of *Cotesia flavipes* (Hymenoptera: Braconidae) parasitizing *Diatraea saccharalis* larvae (Lepidoptera: Crambidae). J. Venom. Anim. Toxins incl. Trop. Dis. 11 (3), 283–298.

Schuld, M., Madel, G., Schmuck, R., 1999. Impact of *Vairimorpha* sp. (Microsporidia: Burnellidae) on *Trichogramma chilonis* (Hymenoptera: Trichgrammatidae), a hymenopteran parasitoid of the cabbage moth, *Plutella xylostella* (Lepidoptera: Yponomeutidae). J. Invertebr. Pathol. 74, 120–126.

Siegel, J.P., Maddox, J.V., Ruesink, W.G., 1986. Impact of *Nosema pyrauta* on a braconid, *Macrocentrus grandii*, in central Illinois. J. Invertebr. Pathol. 47, 271–276.

Thomson, H.M., 1958. The effects of a microsporidian parasite of the spruce budworm, *Choristoneura fumiferana* (Clem.), on two internal hymenopterous parasites. Can. Entomol. 90, 694–696.

Thomson, H.M., 1960. A list and brief description of the microsporidia infecting insects. J. Invertebr. Pathol. 2, 346–385.

Undeen, A.H., Vávra, J., 1997. Research Methods for Entomopathogenic Protozoa. In: Lacey, L.A. (Ed.), Manual of Techniques in Insect Pathology. Academic Press, NY, pp. 117–151.

União dos Produtores de Bioenergia (UPON). 2012. Evolução da produtividade da cana-de açúcar: safras 2005/2006 a 2010/2011. <http://www.udop.com.br/download/estatistica/area_cultivada/01mar12_evolucao_produtividade.pdf>. Accessed on March 14, 2012.

Wittner, M., Weiss, L.M., 1999. The Microsporidia and Microsporidiosis. ASM Press, Washington DC, p. 553.