

# The Use of Meconia to Nondestructively Detect Sublethal Infections in Heliothines (Lepidoptera: Noctuidae)

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**ABSTRACT** The utility of using meconia to nondestructively detect entomopathogens of lepidopterous heliothines was examined. Early-instar tobacco budworm [*Heliothis virescens* (F.)] or cotton bollworm [*Helicoverpa zea* (Boddie)] larvae were inoculated with cytoplasmic polyhedrosis virus (CPV), *Serratia marcescens* Bizio, or *Nosema heliothidis* Lutz and Splendor, and the presence of each of the entomopathogens in adults and the meconia discharged during adult eclosion was determined. As the dose of CPV occlusion bodies and *N. heliothidis* spores but not *S. marcescens* cells ingested by larvae increased, a greater number of both adults and meconia were infested with the entomopathogens. For all three entomopathogens, no difference was observed between males and females for any of the parameters tested. The accuracy of the meconium method for predicting the presence of the entomopathogens in the adults (i.e., number of individuals in which meconia and adults were both positive, or meconia and adults were both negative) was  $\geq 92\%$  for CPV, and  $\geq 79\%$  for *S. marcescens* and *N. heliothidis*. Very few false negative predictions (i.e., the meconium was negative but the adult was positive) were observed for CPV ( $\leq 1\%$ ). The prevalence of false negative predictions ranged from 2 to 9%, and 5 to 21% for *S. marcescens* and *N. heliothidis*, respectively. The prevalence of false positive predictions (i.e., the meconium was positive but the adult was negative) was  $\leq 7\%$  for CPV,  $\leq 13\%$  for *S. marcescens*, and 0% for *N. heliothidis*. The results of this study demonstrate that although not absolute, the meconium method will be an efficacious method to detect nondestructively entomopathogens causing sublethal infections in heliothines, and possibly other insects, and thereby facilitate the rearing of specific pathogen free insects.

**KEY WORDS** Chronic infection, disease, SPF, technique

INSECT REARING IS ESSENTIAL to many aspects of entomological research and technology, and the production of insects of high quality is of paramount importance (Cohen 2001). *Heliothis virescens* (Fabricius) (tobacco budworm), and *Helicoverpa zea* (Boddie) (cotton bollworm) are major economic pests of agricultural crops such as cotton and corn in the United States. Furthermore, mass-reared *H. virescens* and *H. zea* are used to rear ectoparasites, produce microsporidian spores and nuclear polyhedrosis viruses, test for resistant plant cultivars, and develop efficacious biological, chemical and cultural control methods. Cultured lepidopterans also are now being used to produce recombinant proteins of importance to the agricultural and pharmaceutical industries (e.g., Hughes and Wood 1998).

Arguably, the greatest threat to insectary-reared insects does not come from highly virulent pathogens that cause conspicuous disease outbreaks, but rather from less virulent pathogens that cause chronic infec-

tions because disease can easily escape detection. Because chronically infected insects are almost always inferior to noninfected individuals, results based on the use of these insects are often inaccurate, and in some instances, the utilization of these insects will lead to erroneous conclusions. Often the signs of disease are minimal under optimal rearing conditions, but when chronically infected insects are exposed to a stressor (e.g., mechanical damage, environmental or nutritional stress), a disease outbreak can rapidly occur thereby threatening the entire colony with little warning. This is a common scenario in insectaries, and emphasizes the importance of detection and management of chronic infections by entomopathogens.

A number of entomopathogens cause chronic infections in reared insects, and sublethal infections by cytoplasmic polyhedrosis virus (CPV), *Serratia marcescens* Bizio, and *Nosema heliothidis* Lutz and Splendor have been shown to seriously compromise the quality of reared insects (Lipa 1968, Simmons and Sikorowski 1973, Gaugler and Brooks 1975, Sikorowski and Thompson 1979, Thompson and Sikorowski 1979, Sikorowski and Lawrence 1998, Becnel and Andreadis 1999, Sikorowski et al. 2001, Inglis and Lawrence

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2001). Mammals used for scientific purposes are certified as specific pathogen-free (SPF), yet similar standards are not currently used for insects despite the debilitating effects of entomopathogens on insectary-reared insects.

The primary goal of disease management in insectaries is prevention, and this most often involves the early detection and elimination of infected insects from a colony. For example, quarantine of field-collected insects followed by selection of healthy individuals before they are incorporated into the laboratory colony is one of the oldest and best methods for preventing the introduction of pathogen into a colony or the elimination of a pathogen from a colony once it has been detected (e.g., Hamm et al. 1971). The success of the quarantine strategy is reliant on the accurate detection of infection by entomopathogens. In contrast to mammals, the selection strategy primarily involves the destructive sampling of females after oviposition is completed (i.e., if the female is positive for infection, or if she and/or her offspring exhibit abnormal traits, all her offspring are destroyed). This is an indirect method, it is labor intensive and therefore, costly, and can result in the destruction of large numbers of insects, many of them free of infection. The ability to nondestructively detect entomopathogens would facilitate the selection of entomopathogen-free individuals and aid in the designation of insects as SPF. Because insects do not produce immunoglobulins, there are few methods currently available to nondestructively sample insects for entomopathogens.

Many holometabolous insects produce a "meconium" after pupation. Very little is known about the ontogeny of the meconium, but it is believed to contain the waste products of pupal metabolism such as uric acid, which accumulate in the rectum and are discharged when the adult emerges (Chapman 1998). We have used meconia to qualitatively detect CPV (Sikorowski et al. 1971, 1973, Bong and Sikorowski 1991a, b, Sikorowski and Lawrence 1994), *S. marcescens* (Sikorowski and Lawrence 1998, Inglis and Lawrence 2001), and *Nosema* spp. (Sikorowski and Lawrence 1997, Inglis et al. 2003). If the meconium method is to be implemented as a screening method for chronic infections by entomopathogens, it is imperative that the precision of the method is ascertained. Therefore, the objective of this study was to measure the accuracy of using meconia to nondestructively detect sublethal infections of CPV and *S. marcescens* in the tobacco budworm, and *N. heliothidis* in the cotton bollworm.

### Materials and Methods

**Insects.** Eggs of *H. virescens* and *H. zea* were obtained from a healthy colony maintained at the USDA-ARS Biological Control and Mass Rearing Research Unit (BCMRRU) located at Mississippi State University. Eggs were surface-sterilized with formaldehyde (10% vol:vol) for 20 min, and washed 10 times over a period of 2 h in sterile distilled water (Sikorowski and

Goodwin 1985). Eggs were placed on moistened sterile filter paper and allowed to hatch at 24 ( $\pm 2$ ) °C with a photoperiod of 14:10 (L:D) h provided by fluorescent lighting. Neonates were placed on a flash-sterilized wheat germ diet (Shaver and Raulston 1971) containing methyl parabenzoate ( $\approx 0.14\%$  wt:vol) and sorbic acid ( $\approx 0.10\%$  wt:vol). The diet was overlaid with autoclaved corncob grits containing 0.04% (wt:wt) Phaltan and 0.03% (wt:wt) tetracycline hydrochloride.

**Inoculum Preparation.** Occlusion bodies (OB) of the CPV strain were isolated originally from the midgut of an *H. virescens* larva (Simmons and Sikorowski 1973). The isolate of *S. marcescens* was originally isolated from a soybean looper [*Pseuoplusia includens* (Walker)] collected from soybean [*Glycine max* (L.) Merrill] in Holmes County, MS in 1994. The strain of *N. heliothidis* was isolated in 1996 from *H. virescens* reared at DuPont Laboratories, Wilmington, DE.

Occlusion bodies of CPV were proliferated in *H. virescens*. Because the strain of *N. heliothidis* demonstrated high infectivity in *H. zea* but not *H. virescens*, *H. zea* was used to produce spores. For each entomopathogen, larvae (5- to 7-d-old) were allowed to ingest  $\approx 1 \times 10^5$  CPV OB or *N. heliothidis* spores placed on the surface of cotton leaf pieces. Larvae were then maintained on the wheat germ diet as described previously. To obtain CPV OB, the alimentary canals were removed and homogenized in distilled water according to the method of Hosaka and Aizawa (1964). The OB were subsequently concentrated by centrifugation in distilled water. To obtain *N. heliothidis* spores, a Potter-Elvehjem tube was used to homogenize older infected larvae in sterile distilled water, and liberated spores were concentrated by centrifugation. Both OB and microsporidian spores were stored at  $-20^\circ\text{C}$  until used; short-term storage at  $-20^\circ\text{C}$  was found to have no effect on infectivity of either CPV or *N. heliothidis* (data not presented). Immediately before use, a hemocytometer was used to estimate densities of OB and spores per ml. Densities were adjusted with distilled water ( $10^3$ - $10^6$  OB per ml and  $10^3$ - $10^9$  spores per ml), and adjusted densities were confirmed with a hemocytometer.

To obtain inoculum of *S. marcescens*, the bacterium was grown at 24 ( $\pm 1$ ) °C on a medium consisting of 5 g of glucose, 1 g of casein hydrolysate, 1 g of yeast extract, 7 g of  $\text{K}_2\text{HPO}_4$ , 3 g of  $\text{KH}_2\text{PO}_4$ , 1 g of  $(\text{NH}_4)_2\text{SO}_4$ , 0.1 g of  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , and 15 g of agar in 1 liter of water (ATCC medium 1399). After  $\approx 24$  h, bacterial cells were aseptically removed from the surface of the medium, suspended in sterile distilled water, and stored at 5 °C. To quantify the density of cells per ml, the original suspension was serially diluted in a 10-fold dilution series, 100  $\mu\text{l}$  of the suspension was spread on medium 1399, the cultures were incubated at 24 °C, and the number of colony forming units producing red pigmentation were enumerated after 24 h. The strain of *S. marcescens* used in the experiment consistently produces prodigiosin, a nondiffusible water insoluble red pigment (Holt et al. 1994). The cell suspension was then adjusted to  $10^3$ ,  $10^4$ ,  $10^6$ , or  $10^8$

cells per ml in sterile distilled water. To ensure a high level of virulence, the *S. marcescens* isolate was recovered from the hemolymph of a previously healthy *H. virescens* larva that ingested the bacterium just before proliferation on medium 1399.

**Inoculation and Maintenance of Insects.** Five-d-old larvae were placed on cotton leaf pieces ( $\approx 100 \text{ mm}^2$ ) that had been treated with  $10 \mu\text{l}$  of a suspension containing either CPV OB, *S. marcescens* cells or *N. heliothidis* spores. Control larvae were placed on leaf pieces treated with distilled water alone. For CPV and *S. marcescens*, *H. virescens* larvae were used. For *N. heliothidis*, *H. zea* larvae were used. Before use, all leaf pieces were surface-sterilized in 0.5% sodium hypochlorite for 10 min, rinsed in running tap water for 2 h, followed by a final rinse in sterile distilled water (5 min). Leaf pieces and larvae were placed on sterile moistened paper towel in polystyrene cups (32 ml), and larvae were allowed to consume the leaf pieces for 24–48 h. Larvae not consuming the entire leaf piece were eliminated from the experiment. After ingestion of the leaf pieces, larvae were transferred to clean polystyrene cups and starved for a 24 h period; the starvation period was included to permit the elimination of unbound pathogens from the digestive system. After the starvation period, individual larvae were placed on freshly prepared artificial diet in a polystyrene cup.

Larvae were maintained at  $24^\circ\text{C}$  with a photoperiod of 16:8 (L:D) h until pupation ( $\approx 10$ –12 d). Pupae were then removed from the cups, sexed, and surface-sterilized in 0.5% sodium hypochlorite for 5 min followed by two rinses in sterile distilled water. Surface-sterilized pupae were allowed to dry briefly on autoclaved paper towelling, they were then transferred to clean polystyrene cups, and maintained at  $24^\circ\text{C}$  with a photoperiod of 16:8 (L:D) h until eclosion. Within 24 h of emergence, adults were aseptically removed from the polystyrene cups and placed in a new cup. Very few larvae ( $<2\%$ ) inoculated with CPV and *N. heliothidis* died. A slightly higher prevalence of mortality ( $<10\%$ ) was observed in *H. virescens* larvae inoculated with *S. marcescens* at the higher doses.

**Detection of Entomopathogens in Meconia and Adults.** The meconium remaining in each cup after adult eclosion was aseptically homogenized in a small quantity of sterile distilled water ( $\approx 50 \mu\text{l}$ ). To detect the presence of *N. heliothidis* and CPV, the homogenate was smeared onto a glass slide with a flat toothpick, and allowed to air dry. The slide was then heated to  $40^\circ\text{C}$  and covered with a solution of Buffalo Black for 5 min with care taken not to allow the staining solution to dry on the slide during this period. After the incubation period, the slide was removed from the slide warmer and was dipped in tap water for 5 s. The specimen was then allowed to dry, and was examined without a coverslip with an oil immersion objective at a magnification of 1000 times. Using this method, CPV OB and microsporidian spore cell walls stain navy blue while the background remains light blue; the Buffalo Black staining solution dissolves uric acid thereby preventing any confusion in distinguishing CPV OB from

uric acid crystals (Sikorowski et al. 1971). A sample was deemed positive if  $\geq 10$  arbitrarily selected field of views  $\geq 10$  spores or OB. However, extensive numbers of spores or OB ( $>30$ ) were observed in the vast majority of positive samples.

To detect the presence of *S. marcescens* in the meconium, the homogenate was streaked onto ATCC medium 1399 and a semiselective *Serratia* medium (CT Agar) (Starr et al. 1976). Cultures were incubated at  $28^\circ\text{C}$ , and the presence of red-pigmented colonies were recorded after 24–48 h. Throughout the experiment, arbitrarily selected colonies producing the red pigment were isolated, streaked for purity, and a Microscan identification system (Baxter Travenol Laboratories, Inc., Deerfield, IL) was used to confirm their identity (Baxter Travenol Laboratories, Inc.).

Because it was not possible to aseptically remove the alimentary canals of adults, entire insects were homogenized in sterile distilled water. The homogenate was processed for CPV, *N. heliothidis*, and *S. marcescens* as described previously for meconia. To eliminate the possibility that CPV OB and *N. heliothidis* spores may have been associated with frass or remained within the lumen of the alimentary canal, an adult was only designated as positive if extensive numbers of OB or spores ( $>30$  per field of view) were observed in multiple fields of view ( $>10$ ).

**Statistical Analyses.** The experiments were arranged as randomized complete block designs with three to four replicates conducted on separate occasions. Dependent variables included: (1) the number of positive meconia; (2) number of positive adults; (3) accuracy of the method (i.e., number of individuals in which meconia and adults were both positive, or meconia and adults were both negative); (4) number of false negatives (i.e., the meconium is negative but the adult is positive); and (5) number of false positives (i.e., the meconium is positive but the adult is negative). The prevalence of false positive and negative predications were calculated based on total individuals (i.e., positive and negative for infection), and for only individuals that were positive for infection. In all instances, the univariate procedure of SAS (SAS Institute Inc. 1999) was used to ensure normality (using residual values). Initially, each test entomopathogen was analyzed separately with the mixed procedure of SAS as an unbalanced factorial experiment with two levels of sex and four to five levels of dose; replicate was treated as a random variable. In the absence of a significant difference between males and females, the data for sexes was combined. The prevalence of infestation of meconia and adults (i.e., substrate) were compared with the mixed procedure of SAS as an unbalanced factorial experiment with two levels of substrate and four to five levels of dose. The accuracy of the meconium method, and prevalence of false predications were analyzed with the mixed procedure of SAS as a one-way analysis of variance (ANOVA) (with and without the control treatment). In conjunction with a significant *F* test, the least square means function of SAS was used to compare means.

**Results**

**Cytoplasmic Polyhedrosis Virus.** A total of 450 (232 males and 218 females) individuals of *H. virescens* were included in the experiment. Substantial numbers of OB were observed in the homogenates of meconia and adults that were positive for CPV. In no instance were CPV OB observed in meconia or adults originating from larvae not inoculated with the virus ( $n = 119$ ). No difference ( $F \leq 0.7$ ;  $df = 1, 17$ ;  $P \geq 0.63$ ) was detected between males and females for any of the parameters measured, and the results for males and females were constant across dose ( $F \leq 0.7$ ;  $df = 4, 17$ ;  $P \geq 0.63$ ). The data for males and females were combined for all subsequent analyses. The prevalence of infestation of adults and meconia increased ( $F = 25.0$ ;  $df = 4, 17$ ;  $P < 0.001$ ) with the dose of spores ingested by larvae (Fig. 1A). No difference ( $F = 0.2$ ;  $df = 1, 17$ ;  $P = 0.65$ ) was observed in the levels of infestation between adults and meconia across dose. The accuracy of the meconium method for predicting the presence of CPV OB in adults (i.e., number of individuals in which meconia and adults were both positive, or meconia and adults were both negative) ranged from  $92.0 \pm 1.9\%$  to  $100\%$ . The prevalence of false positive predictions (i.e., meconium was positive but adults were negative) ranged from  $0\%$  to  $7.1 \pm 3.1\%$  (Fig. 2A). Only in one instance was a false negative prediction (i.e., meconium was negative but the adult was positive) observed. No effect ( $F \leq 1.6$ ;  $df = 3, 5$ ;  $P \geq 0.31$ ) of dose on either the prevalence of false positive or false negative predictions was detected. Analysis of only individuals positive for CPV ( $n = 352$ ) resulted in an overall (i.e., across doses) prevalence of false positive and false negative predictions of  $4.6 \pm 0.5\%$  and  $0.2 \pm 0.2\%$ , respectively.

**Serratia marcescens.** A total of 644 individuals (341 females and 303 males) were included in the experiment. In no instance was *S. marcescens* isolated from meconia or adults originating from larvae not inoculated with the bacterium ( $n = 183$ ). No difference ( $F \leq 0.11$ ;  $df = 1, 16$ ;  $P \geq 0.74$ ) was observed between the *Serratia* medium and medium 1399 for any of the parameters tested. Nor was there a significant interaction ( $F \leq 0.03$ ;  $df = 4, 16$ ;  $P \geq 0.99$ ) between medium and dose, and data for the *Serratia* medium alone are presented. All of the red-pigmented colonies arbitrarily selected were identified as *S. marcescens*. Limited growth of *S. marcescens* from meconia and/or adults was observed on the *Serratia* medium in some instances, and despite the limited amount of growth, these samples were designated positive for the bacterium.

No difference ( $F \leq 0.18$ ;  $df = 1, 16$ ;  $P \geq 0.67$ ) between males and females was detected for any of the parameters measured, and the results for males and females were constant across dose ( $F \leq 0.75$ ;  $df = 4, 16$ ;  $P \geq 0.57$ ). Therefore, the data were combined across the sexes for all subsequent analyses. Dose influenced ( $F = 32.7$ ;  $df = 4, 16$ ;  $P < 0.001$ ) the prevalence of infestation of adults and meconia (Fig. 1B). However, with the exception of the control treat-

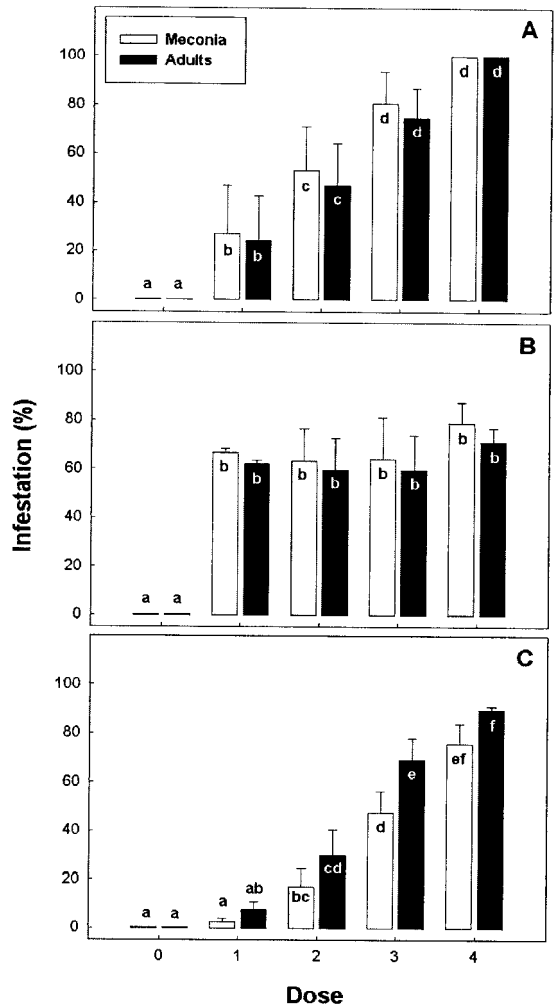


Fig. 1. Infestation (%) of meconia and adult heliothines originating from larvae ingesting varying doses of entomopathogens. (A) *H. virescens* ingesting 0 (control),  $10^1$  (dose 1),  $10^2$  (dose 2),  $10^3$  (dose 3), and  $10^4$  (dose 4) cytoplasmic polyhedrosis virus (CPV) occlusion bodies. (B) *H. virescens* ingesting 0 (control),  $10^1$  (dose 1),  $10^2$  (dose 2),  $10^4$  (dose 3), and  $10^6$  (dose 4) *S. marcescens* cells. (C) *H. zea* ingesting 0 (control),  $10^3$  (dose 1),  $10^4$  (dose 2),  $10^5$  (dose 3), and  $10^6$  (dose 4) *N. heliothidis* spores. Bars represent standard errors ( $n = 3$ ), and bars not accompanied by the same letter are significantly different ( $P \leq 0.05$ ).

ment ( $P < 0.001$ ), no effect ( $P \geq 0.08$ ) of dose on the prevalence of infestation for adults and meconia was observed. No difference ( $F = 0.74$ ;  $df = 1, 16$ ;  $P = 0.40$ ) was observed in the levels of infestation between adults and meconia across dose. The accuracy of the meconium method for predicting the presence of *S. marcescens* in adults ranged from  $78.5 \pm 5.9\%$  to  $90.5 \pm 0.2\%$ . The prevalence of false positive predictions ranged from  $7.1 \pm 0.2$ – $12.7 \pm 6.0\%$ , and the prevalence of false negative reactions ranged from  $2.4 \pm 0.1\%$  to  $8.8 \pm 5.4\%$  (Fig. 2B). No effect ( $F \leq 0.73$ ;  $df = 3, 5$ ;  $P \geq 0.57$ ) of dose was observed on either the prevalence of

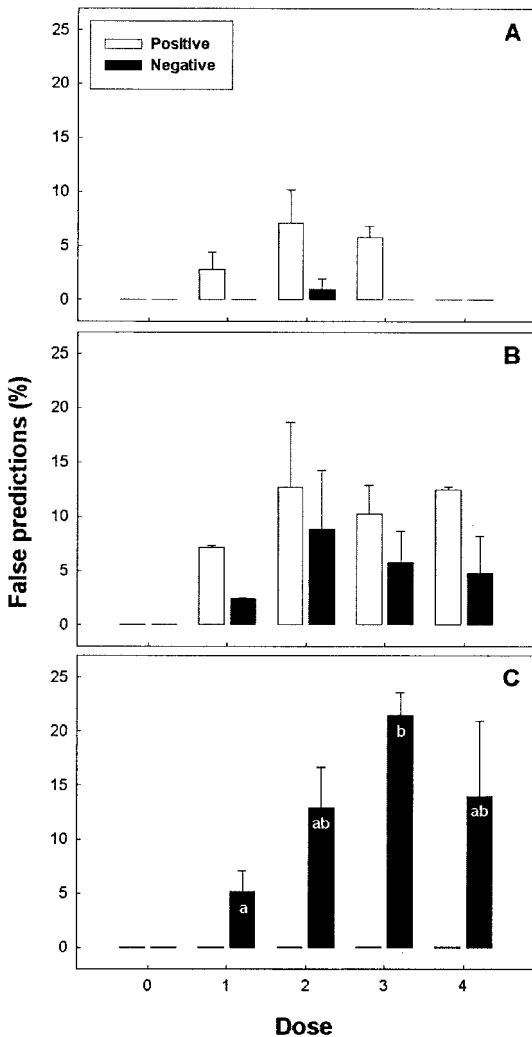


Fig. 2. Prevalence (%) of false positive (i.e., the meconium was positive but the adult was negative) and false negative (i.e., the meconium was negative but the adult was positive) predictions in heliothines ingesting varying doses of entomopathogens. (A) *H. virescens* ingesting 0 (control),  $10^1$  (dose 1),  $10^2$  (dose 2),  $10^3$  (dose 3), and  $10^4$  (dose 4) cytoplasmic polyhedrosis virus (CPV) occlusion bodies. (B) *H. virescens* ingesting 0 (control),  $10^1$  (dose 1),  $10^2$  (dose 2),  $10^4$  (dose 3), and  $10^6$  (dose 4) *S. marcescens* cells. (C) *H. zea* ingesting 0 (control),  $10^3$  (dose 1),  $10^4$  (dose 2),  $10^5$  (dose 3), and  $10^6$  (dose 4) *N. heliothidis* spores. Bars represent SE ( $n = 3$ ), and bars (i.e., false negative predictions for *N. heliothidis*) not accompanied by the same letter are significantly different ( $P \leq 0.05$ ).

false positive or false negative predictions. Analysis of only individuals positive for *S. marcescens* ( $n = 590$ ) resulted in an overall (i.e., across doses) prevalence of false positive and false negative predictions of  $9.7 \pm 3.2\%$  and  $4.9 \pm 2.5\%$ , respectively.

***Nosema heliothidis*.** A total of 454 (218 males and 236 females) individuals of *H. zea* were included in the experiment. Substantial numbers of spores were ob-

served in the homogenates of meconia and adults that were positive for *N. heliothidis*. In no instance were *N. heliothidis* spores observed in meconia or adults originating from larvae not inoculated with the microsporidian ( $n = 105$ ). No difference ( $F \leq 2.6$ ;  $df = 1, 23$ ;  $P \geq 0.12$ ) was observed between males and females for any of the parameters measured, and the results for males and females were constant across dose ( $F \leq 1.2$ ;  $df = 4, 23$ ;  $P \geq 0.33$ ). The prevalence of infestation of adults and meconia increased ( $F = 80.3$ ;  $df = 4, 23$ ;  $P < 0.001$ ) with the dose of spores ingested by larvae (Fig. 1C). A greater ( $F = 10.0$ ;  $df = 1, 23$ ;  $P = 0.004$ ) number of adults were infested relative to meconia across dose. The accuracy of the meconium method for predicting the presence of *N. heliothidis* in adults ranged from  $78.5 \pm 2.1\%$  to  $94.8 \pm 1.9\%$ . No false positive predictions were observed. In contrast, the prevalence of false negative predictions ranged from  $5.2 \pm 1.9$ – $21.5 \pm 2.1\%$  (Fig. 2C). The number of spores ingested by larvae weakly affected ( $F = 7.7$ ;  $df = 3, 7$ ;  $P = 0.047$ ) the prevalence of false negative results; at a dose of  $10^6$  spores per ml, less ( $P = 0.008$ ) false negatives relative to the  $10^8$  dose were observed. Analysis of only individuals positive for *N. heliothidis* ( $n = 241$ ) resulted in an overall (i.e., across doses) prevalence of false negative predictions of  $22.7 \pm 6.3\%$ .

## Discussion

The “meconium method” has been used in our laboratory to qualitatively detect *S. marcescens*, *N. heliothidis*, and CPV (Sikorowski et al. 1971, 1973, Sikorowski and Lawrence 1994, 1997, 1998, Inglis and Lawrence 2001; Inglis et al. 2003). In the current study, we confirmed that CPV OB were excreted in meconia of *H. virescens*. Furthermore, we found that the meconium method was very accurate in predicting infections by CPV ( $\geq 92\%$ ), and overall we observed  $<1\%$  false negative predictions. In contrast to CPV, the meconium method was less accurate in predicting infections by *S. marcescens* and *N. heliothidis* ( $\geq 79\%$ ). We observed that the meconium method resulted in 5% and 23% overall false negative predictions for *S. marcescens* and *N. heliothidis*, respectively. We also detected a number of false positive predictions for CPV (5%) and *S. marcescens* (10%) but not for *N. heliothidis*. Although we were unable to detect either CPV or *S. marcescens* in adults in these instances, their presence in meconia indicates that these insects are not SPF. In contrast, false negative results are of paramount concern to insect rearers (i.e., falsely determining that an insect is free of an entomopathogen). Based on the prevalence of false negative predictions, our results indicate that the meconium method is  $>99\%$  effective in predicting infection by CPV in *H. virescens*, 95% effective in detecting *S. marcescens* in *H. virescens*, and 77% effective in predicting infections by *N. heliothidis* in *H. zea*. There is no doubt that these levels of predictability would greatly facilitate the selection of SPF individuals in rearing programs for heliothines.

Reasons for the higher predictability of the meconium method for CPV relative to *S. marcescens* and *N. heliothidis* are speculative. The ontogeny of meconium is largely unknown, but meconia do contain the waste products of larval and pupal metabolism. In Lepidoptera for example, uric acid (i.e., a waste product of protein and purine metabolism) accumulates in the fat body cells of larvae and pupae, and it or its conversion products (e.g., allantoin or allantoic acid) are transferred to the rectum and are excreted in the meconium when the adult emerges (Chapman 1998). Ommochromes (e.g., xanthommatin), conversion products of tryptophan, also are excreted in the lepidopteran meconium and are responsible for its coloration (Chapman 1998). Other compounds also have been detected in meconia. For example, Sikorowski and Thompson (1985) observed that streptomycin fed to *H. virescens* larvae was present in the tissues and blood, but the antibiotic was subsequently excreted in meconia during transformation from pupae to adults.

How entomopathogens enter meconia is unknown. One possibility is that propagules of entomopathogens are shed into the gut lumen at various stages of the infection process, and they are subsequently excreted in meconia. Infections by CPV in heliothines are restricted to the mid-gut epithelial cells, and infected columnar cells rupture to release OB in the gut lumen 2 or 3 d after infection (Bong and Sikorowski 1991a, b). Infections by *N. heliothidis* also affect the midgut epithelium as well as fat body cells, trachea and gonads of *H. zea* (Lipa 1968, Sikorowski and Lawrence 1997). Microsporidia that infect the alimentary canal are almost always disseminated in feces (Weiser 1961; Becnel and Andreadis 1999), and this is important for the horizontal transmission of many *Nosema* species (e.g., Lewis and Lynch 1978; Lewis and Cossentine 1986). Numerous *Nosema* species also infect the Malpighian tubules (Becnel and Andreadis 1999), and spores released into the lumen of the tubules may subsequently be excreted in meconia with nitrogenous waste products. However, *N. heliothidis* is not restricted to the midgut tissues, and this may explain the higher prevalence of false negatives predictions that we observed relative to CPV.

Very little is known about the mechanisms of pathogenesis by *S. marcescens* in insects. It is well documented that the bacterium can proliferate and kill various insects by septicemia once it has breached the peritrophic matrix (PM), penetrated the midgut, and gained access to the hemocoel (e.g., Sri-Arunotai et al. 1975). However, the site of infection and the fate of *S. marcescens* causing sublethal infections in insects is not well understood. *S. marcescens* produces a chitinase which is thought to facilitate penetration of the PM (e.g., Hüber et al. 1991), and multiplication of the bacterium within midgut cells of lepidopteran larvae has been observed (Tomita and Iwashita 1987). Furthermore, *S. marcescens* is often found in association with midgut tissues (e.g., Demaio et al. 1996), and it is excreted in frass (e.g., Steinhilber 1959). Of the three entomopathogens tested, only *S. marcescens* can proliferate saprotrophically. Similarly to the findings of

the current study, a number of researchers have observed a poor dose-response for *S. marcescens* (e.g., Steinhilber 1959, Sikorowski et al. 2001). We starved larvae after ingestion of the bacterium in an attempt to permit the elimination of unbound pathogens from the digestive system. Nevertheless, the poor dose response that we observed may be due, at least in part, to the bacterium's ability to breach the PM and to proliferate saprotrophically in association with the epithelial cells of the midgut.

To measure infection, we homogenized intact adults (i.e., including the alimentary canal). This made definitive determinations on whether entomopathogens represented infections (i.e., intracellular or intrahemocoelic) or simply propagules associated with the surface of gut tissues or present within the gut lumen a potential problem. We possess considerable experience in the microscopic assessment of *Nosema* and CPV infections in lepidopteran insects, and the large numbers of OB and spores that we observed in the larval homogenates were indicative of infection. Furthermore, we observed that densities of OB and spores in the homogenates were independent of dose (i.e., as many propagules were observed in insects receiving the low and high dose treatments).

Sajap and Lewis (1989) observed that *N. pyrausta* spores ingested by *Chrysoperla carnea* (Neuroptera: Chrysopidae) did not penetrate the gut wall, they remained ungerminated within the gut lumen, and the spores were subsequently excreted in meconia. However, in contrast to lepidoptera, chrysopid larvae possess a blind gut, and there is no passage to the hindgut and elimination of waste (Gaumont 1976). Furthermore, the transit times of ingested food is relatively rapid ( $\approx 1.8$  h) in heliothine larvae (e.g., Broadway 1997), and the midgut epithelium of lepidopteran larvae produces a PM that encloses the food bolus and prevents food particles from coming in contact with the microvilli of the midgut cells (Chapman 1998). The PM also can prevent microorganisms from reaching the epithelial cells of the midgut (Lehane 1997). Cytoplasmic polyhedrosis virus and *N. heliothidis* are biotrophs, and they can only multiply within a host (e.g., if  $10^3$  spores are ingested by a larva, there can only be more spores than this in the homogenates if infection has occurred). Given the collective evidence, it is highly likely that the large numbers of OB and spores that we observed in the adult homogenates emanated from infections. The microbiological method used to detect *S. marcescens* (i.e., based on the ability of the bacterium to grow on an agar medium) provides a qualitative measure biomass clouding conclusions on the status of the pathogen in relation to the host (e.g., representing infection or saprotrophic existence). Nevertheless, the meconium method was very effective for detecting the presence of the bacterium regardless of its association with the insect. This is significant given that *S. marcescens* present at low numbers in an insect can rapidly multiply and incite disease when insect vigor is reduced (e.g., the insect is exposed to a physiological stressor) (Sikorowski and Lawrence 1998).

The meconium method was found to predict the presence of CPV associated with heliothine adults in  $\geq 92\%$  of the cases, and of *S. marcescens* and *N. heliothidis* in  $\geq 79\%$  of the individuals examined. A low prevalence of false negative predictions were observed for CPV (0.2%) and *S. marcescens* (5%). For *N. heliothidis*, the prevalence of false negative results was 23%. Furthermore the technique was rapid, and two workers, one preparing smears and the other examining the smears with a light microscope can check up to 50 smears per hour (Sikorowski and Lawrence 1997). The results of the current study indicate that the meconium method could be a valuable tool for nondestructively detecting CPV, *N. heliothidis*, and *S. marcescens* associated with heliothines. Whether the meconium method will prove useful in the detection of other entomopathogens requires testing, but will no doubt be dependent on characteristics of each individual pathogen and insect. However, elucidation of how meconia is formed, what tissues are involved in its formation, and how entomopathogens enter meconia will facilitate the development of the meconium method for nondestructively detecting a wide range of pathogens of insects, and the development of an SPF designation for reared insects.

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#### References Cited

Becnel, J. J., and T. G. Andreadis. 1999. Microsporidia in insects, pp. 447–501. In M. Wittner [ed.], *The microsporidia and microsporidiosis*. American Society of Microbiology, Washington, DC.

Bong, C.F.J., and P. P. Sikorowski. 1991a. Histopathology of cytoplasmic polyhedrosis virus (Reoviridae) infection in corn earworm *Helioverpa zea* (Boddie) larvae (Insecta: Lepidoptera: Noctuidae). *Can. J. Zool.* 69: 2121–2127.

Bong, C.F.J., and P. P. Sikorowski. 1991b. Presence of polyhedra in the midgut cell nuclei of *Heliothis zea* (Lepidoptera: Noctuidae) infected with cytoplasmic polyhedrosis virus. *J. Invertebr. Pathol.* 57: 294–295.

Broadway, R. M. 1997. Dietary regulation of serine proteinases that are resistant to serine proteinase inhibitors. *J. Insect Physiol.* 43: 855–874.

Chapman, R. F. 1998. *The insects: structure and function*, 4<sup>th</sup> ed. Cambridge University Press, Cambridge, UK.

Cohen, A. C. 2001. Formalizing insect rearing and artificial diet technology. *Am. Entomol.* 47: 198–206.

Demaio, J., C. B. Pumpuni, M. Kent, and J. C. Beier. 1996. The midgut bacterial flora of wild *Aedes triseriatus*, *Culex*

*pipiens*, and *Psorophora columbiae* mosquitoes. *Am. J. Trop. Med. Hyg.* 54: 219–223.

Gaugler, R. R., and W. M. Brooks. 1975. Sublethal effects of infection by *Nosema heliothidis* in the corn earworm, *Heliothis zea*. *J. Invertebr. Pathol.* 26: 57–63.

Gaumont, J. 1976. L'appareil digestif des larves de planipennes. *Ann. Sci. Nat. Zool. Biol. Anim.* 18: 145–249.

Hamm, J. J., R. L. Burton, J. R. Young, and R. T. Daniel. 1971. Elimination of *Nosema heliothidis* from a laboratory colony of the corn earworm (*Heliothis zea*). *Ann. Entomol. Soc. Am.* 64: 624–627.

Holt, J. G., N. R. Krieg, P.H.A. Sneath, J. T. Staley, and S. T. Williams (eds.). 1994. *Bergey's manual of determinative bacteriology*. Williams and Wilkins, Baltimore, MD.

Hosaka, Y., and K. Aizawa. 1964. The fine structure of cytoplasmic polyhedrosis virus of the silkworm *Bombyx mori* Linn. *J. Insect Pathol.* 6: 53–77.

Huber, M., E. Cabib, and L. H. Miller. 1991. Malaria parasite chitinase and penetration of the mosquito peritrophic membrane. *Proc. Natl. Acad. Sci. USA* 88: 2807–2810.

Hughes, P. R., and H. A. Wood. 1998. Production of pharmaceutical and other recombinant proteins in insect larvae. *SIM News* 48: 100–105.

Inglis, G. D., and A. M. Lawrence. 2001. Effects of *Serratia marcescens* on the F<sub>1</sub> generation of laboratory-reared *Heliothis virescens* (Lepidoptera: Noctuidae). *J. Econ. Entomol.* 94: 362–366.

Inglis, G. D., A. M. Lawrence, and F. M. Davis. 2003. Impact of a novel species of *Nosema* on the southwestern corn borer (Lepidoptera: Crambidae). *J. Econ. Entomol.* 96: 12–20.

Lehane, M. J. 1997. Peritrophic matrix structure and function. *Annu. Rev. Entomol.* 42: 525–550.

Lewis, L. C., and J. E. Cossentine. 1986. Season long intraplant epizootics of entomopathogens, *Beauveria bassiana* and *Nosema pyrausta*, in a corn agroecosystem. *Entomophaga* 31: 363–369.

Lewis, L. C., and R. E. Lynch. 1978. Foliar application of *Nosema pyrausta* for suppression of populations of European corn borer. *Entomophaga* 23: 83–88.

Lipa, J. J. 1968. Some observations on *Nosema heliothidis* Luts et Splendore, a microsporidian parasite of *Heliothis zea* (Boddie) (Lepidoptera: Noctuidae). *Acta Protozool.* Pol. 4: 273–278.

Sajap, A. S., and L. C. Lewis. 1989. Impact of *Nosema pyrausta* (Microsporidia: Nosematidae) on a predator, *Chrysoperla carnea* (Neuroptera: Chrysopidae). *Environ. Entomol.* 18: 172–176.

SAS Institute 1999. *User's guide*, version 8.0. SAS Institute, Cary, NC.

Shaver, T. N., and J. R. Raulston. 1971. A soybean-wheat germ diet for rearing of the tobacco budworm. *Ann. Entomol. Soc. Am.* 64: 1077–1079.

Sikorowski, P. P., G. L. Andrews, and J. R. Broome. 1973. Trans-ovum transmission of a cytoplasmic polyhedrosis virus of *Heliothis virescens* (Lepidoptera: Noctuidae). *J. Invertebr. Pathol.* 21: 41–45.

Sikorowski, P. P., J. R. Broome, and G. L. Andrews. 1971. Simple methods for detection of cytoplasmic polyhedrosis virus in *Heliothis virescens*. *J. Invertebr. Pathol.* 17: 451–452.

Sikorowski, P. P., and R. H. Goodwin. 1985. Contaminant control and disease recognition in laboratory colonies, pp. 85–105. In P. Singh and R. R. Moore [eds.], *Handbook of insect rearing*, vol. 1. Elsevier, Amsterdam, The Netherlands.

Sikorowski, P. P., and A. M. Lawrence. 1994. *Heliothis* cytoplasmic polyhedrosis virus and its effect upon microbial

- contaminant-free *Heliothis virescens*. J. Invertebr. Pathol. 63: 56–62.
- Sikorowski, P. P., and A. M. Lawrence. 1997. Major diseases of *Heliothis virescens* and *Helicoverpa zea* in Mississippi fields and insectaries. Miss. Agric. For. Exp. St. Tech. Bull. 218, Mississippi State, MS.
- Sikorowski, P. P., and A. M. Lawrence. 1998. Transmission of *Serratia marcescens* (Enterobacteriaceae) in adult *Heliothis virescens* (Lepidoptera: Noctuidae) laboratory colonies. Biol. Contr. 12: 50–55.
- Sikorowski, P. P., A. M. Lawrence, and G. D. Inglis. 2001. Effects of *Serratia marcescens* on rearing tobacco budworm (Lepidoptera: Noctuidae). Am. Entomol. 47: 51–60.
- Sikorowski, P. P., and A. C. Thompson. 1979. Effects of cytoplasmic polyhedrosis virus on diapausing *Heliothis virescens*. J. Invertebr. Pathol. 33: 66–70.
- Sikorowski, P. P., and A. C. Thompson. 1985. Residual quantities of streptomycin sulfate in hemolymph and tissue of *Heliothis virescens* reared on treated diet. Pharm. Biochem. Behav. 23: 525–528.
- Simmons, C. L., and P. P. Sikorowski. 1973. A laboratory study of the effects of cytoplasmic polyhedrosis virus on *Heliothis virescens* (Lepidoptera: Noctuidae). J. Invertebr. Pathol. 22: 369–371.
- Sri-Arunotai, S., P. P. Sikorowski, and W. W. Neel. 1975. Study of pathogens of the pecan weevil larvae Environ. Entomol. 4: 790–792.
- Starr, M. P., P.A.D. Grimont, F. Grimont, and P. B. Starr. 1976. Caprylate-thallos agar medium for selectively isolating *Serratia* and its utility in the clinical laboratory. J. Clin. Microbiol. 4: 270–276.
- Steinhaus, E. A. 1959. *Serratia marcescens* Bizio as an insect pathogen. Hilgardia 14: 351–380.
- Thompson, A. C., and P. P. Sikorowski. 1979. Effects of *Nosema heliothidis* on fatty and amino acids in larvae and pupae of the bollworm, *Heliothis zea*. Comp. Biochem. Physiol. 63A: 325–328.
- Tomita, K., and Y. Iwashita. 1987. Study on the serological reaction of *Enterobacter* sp. multiplying in the cytoplasm of midgut cells of some lepidopterous larvae. Jpn. J. Appl. Entomol. Zool. 31: 70–75.
- Weiser, J. 1961. Die mikrosporidien als parasiten der insekten. Mongr. Angew. Entomol. 17: 1–149.

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