Juvenile Hormone Analog Technology: Effects on Larval Cannibalism and the Production of *Spodoptera exigua* (Lepidoptera: Noctuidae) Nucleopolyhedrovirus

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ABSTRACT The production of a multiple nucleopolyhedrovirus (SeMNPV) of the beet armyworm, *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae), has been markedly increased by using juvenile hormone analog (JHA) technology to generate a supernumerary sixth instar in the species. In the current study we compared the incidence of cannibalism in *S. exigua* fifth and sixth instars reared at low (two larvae per dish) and a high density (10 larvae per dish). The incidence of cannibalism was significantly higher in fifth instars compared with sixth instars and increased with rearing density on both instars. Infected larvae were more prone to become victims of cannibalism than healthy individuals in mixed groups comprising 50% healthy + 50% infected larvae in both instars reared at high density. Instar had a marked effect on occlusion body (OB) production because JHA-treated insects produced between 4.8- and 5.6-fold increase in OB production per dish compared with fifth instars at high and low densities, respectively. The insecticidal characteristics of OBs produced in JHA-treated insects, as indicated by LD_{50} values, were similar to those produced in untreated fourth or fifth instars. Because JHA technology did not increase the prevalence of cannibalism and had no adverse effect on the insecticidal properties of SeMNPV OBs, we conclude that the use of JHAs to generate a supernumerary instar is likely to be compatible with mass production systems that involve gregarious rearing of infected insects.

KEY WORDS *Spodoptera exigua*, cannibalism, juvenile hormone, nucleopolyhedrovirus production

Cannibalism, or intraspecific predation, is a frequent behavior in many species of Lepidoptera during the larval stage (Pierce 1995, Chapman et al. 2000). Although cannibalism can confer direct fitness benefits in the form of increased development rate, fecundity, or the removal of potential competitors (Joyner and Gould 1985, Church and Sherratt 1996), it also can have profound consequences on insect population dynamics (Reed et al. 1996), the risk of acquiring disease (Rudolf and Antonovics 2007), and the inclusive fitness of cannibals that consume kin (Fox 1975, Polis 1981).

The prevalence of cannibalistic behavior is often density-dependent, with an increasingly higher incidence at higher densities, even when food is not limiting (Polis 1981). Cannibalism may also be stage dependent in certain species of Lepidoptera with later instars often showing a greater tendency to engage in intraspecific predation, compared with their younger and smaller conspecifics, especially when larvae of different stages are enclosed together (Chapman et al. 1999a,b).

Baculoviruses form the basis for many biological insecticides with proven commercial potential (Moscardi 1999). However, one factor that limits their commercial development is the need to produce these viruses in living insects, which requires the maintenance of large insect colonies in mass rearing facilities (Shapiro 1986). Recently, we applied juvenile hormone analog (JHA) technology to the production of a nucleopolyhedrovirus (SeMNPV) of the beet armyworm, *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae). The application of JHA compounds results in multiple changes in the developing insect that have been reviewed in detail (Gilbert et al. 2000, Wilson 2004). Treating fifth instars with methoprene or fenoxycarb resulted in the generation of a supernumerary sixth instar, that when infected by feeding on viral occlusion bodies (OBs), yielded an almost three-fold increase in the number of OBs produced in each larva, with no significant reduction in OB potency, compared with OBs produced in infected fifth instar conspecifics (Lasa et al. 2007).

As SeMNPV is now being produced commercially under the name of Spod-X (Certis USA, Columbia, MD), Vir-ex (Biocolor, Almeria, Spain), and as various products produced by local companies in southern

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and southeast Asia, we examined the influence of applying JHA technology to the prevalence of cannibalism of groups of *S. exigua* supernumerary instars in the laboratory and the impact that cannibalistic behavior has on the production and insecticidal potency of SeMNPV OBs.

Materials and Methods

Insect Colony, JHA, and Virus Strain. *S. exigua* larvae were obtained from a laboratory colony maintained at 25 ± 2°C, 70 ± 5% humidity, and a photoperiod of 16:8 (L:D) h in the Universidad Pública de Navarra, Pamplona, Spain. An artificial diet adapted from Hoffman and Lawson (1964) was used to feed larvae. Fifth instars were obtained from the colony and were individually treated topically with 3 μl of a commercial fenoxycarb product (Zambu 25 W, Agro Artés, Castellón, Spain) that had been diluted to a concentration of 2.5 μg active ingredient (AI)/ml in distilled water. Treated larvae were incubated individually for 5 d on artificial diet until they had molted to the supernumerary sixth instar.

OBs of a Spanish nucleopolyhedrovirus isolate SeMNPV-SP2 (Caballero et al. 1992) were produced by orally inoculating *S. exigua* fourth instars. OBs were collected from insects that subsequently died of polyhedrosis disease, purified by centrifugation (Muñoz et al. 1997), quantified by counting in triplicate in a Neubauer chamber (Hawksley, Lancing, United Kingdom), and stored at 4°C until required.

Supernumerary Instar and Rearing Density Effects on Cannibalism. Groups of fifth and sixth instars were allowed to feed on droplets of 10% (wt:vol) sucrose, 0.001% (vol:vol) fluorella blue food dye, and a concentration of OBs of 4.0×10^5 or 1.0×10^6 OBs/ml, which had been established previously to result in ≈90% infection in fifth and sixth instars, respectively. Larvae that drank the suspension within 10 min were selected for inclusion in the experiment and were placed in petri dishes at densities of either two or 10 larvae per dish. Larvae were assigned to one of six treatments at each density on a random basis according to their condition. These were 1) healthy fifth instars, 2) infected fifth instars, 3) 50% healthy + 50% infected fifth instars, 4) healthy sixth instars, 5) infected sixth instars, and 6) 50% healthy + 50% infected sixth instars. Healthy and infected larvae in mixed treatments were differentiated by marking dorsally with indelible inks that had been found not to affect survival in preliminary tests. Each petri dish contained a piece of semisynthetic diet that represented excess food for the period of the experiment. Dishes were incubated at 28 ± 2°C for 6 d, and the number of surviving larvae was noted daily. We considered that larvae have been cannibalized when observed to be dead and partially or totally consumed. The experiment was performed 20 times for every treatment and density. The number of insects that were cannibalized and the total number of insects tested (n) were used to determine the proportion of cannibalism (p) by fitting generalized linear models using GLIM four with a binomial error structure specified (Numerical Algorithms Group 1993). In the absence of overdispersion, the results of such analyses closely approximate to the chi-square distribution. The errors of binomially distributed means become increasingly asymmetrical as they approach the limits of the distribution (1 or 0). Results are shown as percentages of cannibalism in the corresponding figures.

Supernumerary Instar and Rearing Density Effects on OB Production and Potency. After a 6-d incubation period at 28 ± 2°C, the number of dead larvae was noted, and the disappearance of infected and healthy insects in mixed treatments was similarly recorded. Petri dishes were placed at −20°C until completely frozen, which facilitated collection of infected larvae that tended to become liquefied at room temperature. Healthy larvae that may have acquired an infection in the mixed treatments were not collected. Frozen cadavers of the infected larvae were individually weighed and placed in 50-ml tubes containing 30 ml of sterile distilled water. The suspension was filtered through fine steel gauze to remove insect debris. The total number of OBs produced in each dish was determined in triplicate using a Neubauer improved counting chamber under phase contrast microscopy at 400× magnification. The procedure was performed 20 times at the lower density and 10 times at the higher density. OB production and weights of frozen larvae were normalized by log transformation and were subjected to analysis of variance (ANOVA) in SPSS, version 15.0 (SPSS Inc., Chicago, IL).

The insecticidal activity of OBs collected was compared with the original inoculum produced in fourth-instar individualized larvae. Pathogenicity was determined in *S. exigua* second instars from the laboratory colony by using a modified droplet bioassay technique (Hughes and Wood 1996). Late first instars were starved for 12 h at 25 ± 2°C and allowed to molt to the next instar over period of 10 h. Groups of 30 larvae were allowed to feed on droplets of 10% sucrose, 0.001% fluorella blue food dye and one of five concentrations of OBs in the range of 3.03×10^3 to 2.45×10^5 OBs/ml, calculated previously to result in mortalities of between 10 and 90%. Control larvae were treated identically but fed on a solution of sucrose and food dye alone. For each concentration, 25 larvae that had ingested the OB suspension within 10 min were placed individually in the cells of a tissue culture plate containing diet and incubated at 25 ± 2°C. Mortality was noted at 5 d postinoculation. The bioassay was performed three times. Results were subjected to logit regression in GLIM with a binomial error distribution specified (Numerical Algorithms Group 1993).

Results

Supernumerary Instar and Rearing Density Effects on Cannibalism. The prevalence of cannibalism (Fig. 1A and B) was significantly higher among larvae in the high density treatments compared with the low density treatments ($\chi^2 = 40.5$, df = 1, $P < 0.001$). At a density of two larvae per dish (Fig. 1A) losses due to

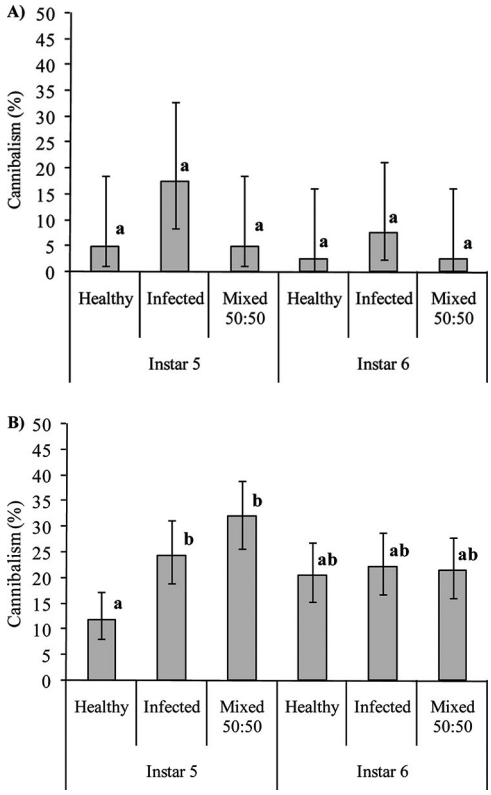

Fig. 1. Percentage of cannibalism of *S. exigua* larvae in the fifth and sixth instar reared at densities of two larvae per dish (A) and 10 larvae per dish (B). Vertical bars indicate the binomially-distributed 95% confidence interval (CI). Columns labeled with identical letters did not differ significantly for comparisons between treatments ($P > 0.05$; Generalized Linear Model).

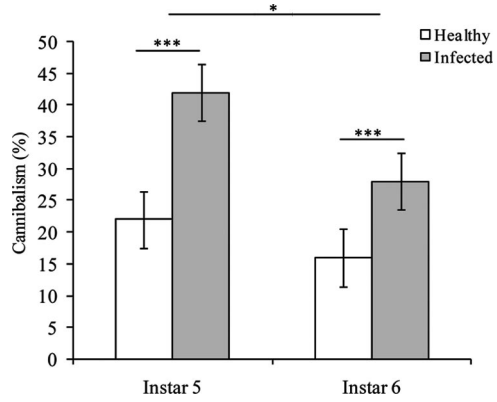

Fig. 2. Percentage of cannibalism observed in healthy and infected fifth and sixth instars reared at a density of 10 larvae per dish in groups consisting of 50% healthy + 50% infected. Vertical bars indicate SE. Asterisks next to horizontal bars placed above columns indicate statistical differences between the corresponding treatments as determined by fitting generalized linear models with a binomial error structure (*, $P < 0.05$; ***, $P < 0.001$).

cannibalism not differ significantly in any treatment ($\chi^2 = 5.9$, df = 5, $P = 0.31$). In contrast, at a density of 10 larvae per dish (Fig. 1B) cannibalism was significantly higher in the fifth-instar treatments involving virus infected and a mixture of healthy + infected larvae, compared with groups comprising healthy fifth instars alone ($\chi^2 = 25.3$, df = 5, $P < 0.001$), whereas all treatments involving sixth instars were intermediate for cannibalism (Fig. 1B). No virus infections were observed in any of the healthy larvae of either instar at both densities.

A closer examination of the patterns of cannibalism in the mixed 50% healthy + 50% infected treatments (Fig. 2) revealed that at high density, the percentage of insects that fell victim to cannibalism was significantly higher among infected larvae of both instars ($\chi^2 = 12.40$, df = 1, $P < 0.001$). Host instar also had a significant effect with sixth instars showing a reduced prevalence of cannibalism compared with fifth instars ($\chi^2 = 5.84$, df = 1, $P = 0.016$). In contrast, the prevalence of cannibalism was too low in the low density treatments to permit a reliable analysis; in total two infected larvae fell victim to cannibalism in the fifth-instar treatment, whereas one infected larva was cannibalized in the sixth instar treatment.

Supernumerary Instar and Rearing Density Effects on OB Production and Potency. The final weight of sixth instars (Fig. 3A) was between 3- and 3.5-fold greater than that of fifth instars at both densities ($\chi^2 = 342.7$, df = 7, $P < 0.001$). As a result, the total yield of OBs per larva (Fig. 3B) was 4.8–5.6-fold higher in infected sixth instars from the 100% infected treatment and the 50% infected + 50% healthy treatment (in which only OBs from initially infected insects were quantified) at densities of two larvae per dish ($\chi^2 = 58.7$, df = 3, $P < 0.001$) and 10 larvae per dish ($\chi^2 = 29.6$, df = 3, $P < 0.001$). OB yield per mg of body weight (±SE) ranged between $8.56 \times 10^6 \pm 1.47 \times 10^6$ and $1.07 \times 10^7 \pm 1.18 \times 10^6$ but did not differ significantly between instars at a density of two larvae per dish ($F = 0.97$; df = 3, 79; $P = 0.41$) or 10 larvae per dish ($F = 1.25$; df = 3, 39; $P = 0.31$), irrespective of whether groups of larvae were all infected or whether groups initially comprised mixtures of healthy + infected larvae. As expected given the number of insects present, the total production of OBs per dish was highest in high density treatments ($\chi^2 = 103.4$, df = 7, $P < 0.001$), with the highest mean ± SE value of $3.43 \times 10^{10} \pm 1.96 \times 10^9$ OBs per dish in dishes containing solely infected sixth instars (Fig. 3C). This represented a 4.8-fold increase per dish compared with the OB production in fifth instars at an initial density of 10 larvae per dish. Similarly, at an initial density of two larvae per dish, overall OB production was 5.8-fold greater in sixth instars compared with fifth instars.

The potencies of OBs produced in individualized fourth instars represented a control treatment (Table 1). There was no significant interaction between dose and treatment ($\chi^2 = 2.25$, df = 2, $P = 0.32$) such that regressions could be fitted in parallel with a common slope (±SE) of 0.666 ± 0.048. The estimated LD_{50} values did

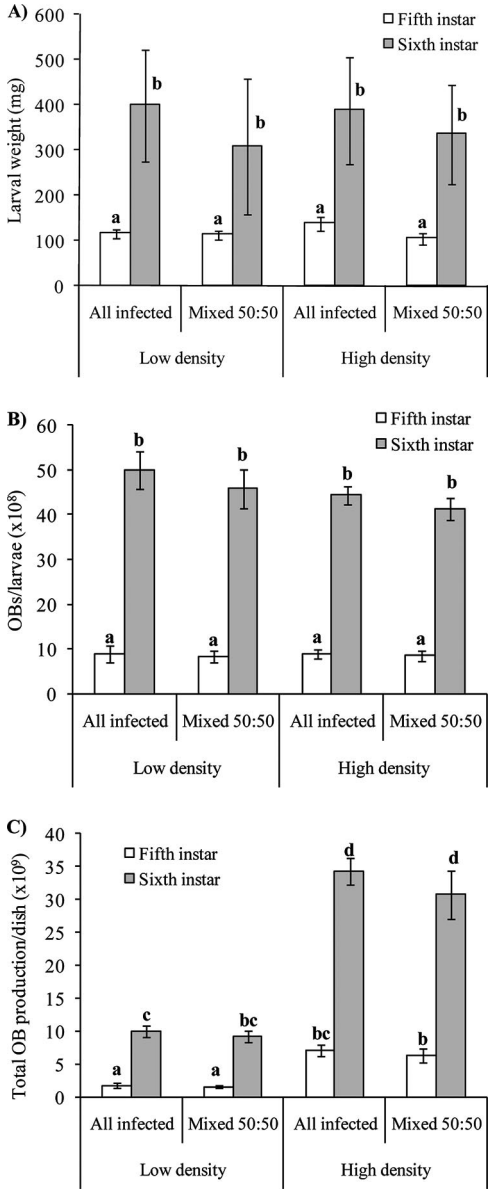

Fig. 3. Density and host instar effects on larval weight (milligrams) at death (6 d postinfection; A), OB production per larva (B), and total OB production per petri dish (C). Vertical bars indicate SE. In all cases columns labeled with identical letters did not differ significantly for comparisons between treatments (ANOVA; $P > 0.05$).

not differ significantly between instars with values of 10.0, 8.6, and 12.9 for the fourth instar controls, fifth, and sixth instars, respectively. No evidence of overdispersion was observed in data sets, and no mortality by virus infection was registered in control insects.

Discussion

Cannibalism has been reported widely in many species of lepidopterous larvae. This study demonstrates that cannibalism was a frequent behavior in a laboratory population of *S. exigua* although to a lesser extent than observed in species such as *Heliothis virescens* (F.) (Gould et al. 1980), *Spodoptera frugiperda* (J.E. Smith) (Chapman et al. 1999a, b), or *Helicoverpa zea* (Boddie) (Joyner and Gould 1985). As expected, the frequency of cannibalism increased with increasing density. Many other studies have demonstrated the relationship between the incidence of cannibalism and the degree of crowding (Chapman et al. 1999b). In fact, higher frequency of cannibalism occurs when hosts are exposed to overcrowding or nutritional stress (Vasconcelos 1996), which could have an effect on pathogen transmission. High-density morphs differ from conspecifics reared at low-densities in a variety of features including color, behavior, and developmental time (Tojo et al. 1985, Pener 1991). These changes are generally assumed to have evolved in response to increased intraspecific competition for food or density-dependent predation pressures. Moreover, larvae develop more quickly but attain smaller size at pupation and are more susceptible to disease when reared at high than at low densities (Goulson and Cory 1995).

Table 1. Logit regression analysis of SeMNPV OBs[a] produced in fifth instars or supernumerary sixth instars compared with control OBs produced in fourth instars

Source of OBs (host stage)	Intercept ± SE	LD_{50}[b] (OBs per larvae)	95% CI Upper	95% CI Low
Fourth instar (control)	1.53 ± 0.16	10.0a	12.5	8.0
Fifth instar	1.42 ± 0.17	8.6a	10.6	6.8
Sixth instar	1.70 ± 0.17	12.9a	16.1	10.3

[a] OBs were bioassayed by feeding to *S. exigua* second instars.
[b] LD_{50} values followed by identical letters did not differ significantly as determined by pairwise *t*-tests ($P > 0.05$).

The frequency of cannibalism was also affected by the stage of the individuals involved; cannibalism was higher in fifth instars compared with supernumerary sixth instars generated by JHA treatment. JHAs tend to cause severe disruptions in the organization of the central nervous system, salivary glands and musculature in a dose-dependent manner (Restifo and Wilson 1998). In consequence, marked changes occur in the physiological and developmental behavior of the individuals treated. We observed that sixth instars were noticeably less agile and apparently less willing to engage in aggressive interactions than their fifth-instar conspecifics.

Cannibalism has been reported as a viable route of horizontal transmission of several viruses in natural and laboratory populations of Lepidoptera, including nucleopolyhedroviruses in *Helicoverpa armigera* (Hübner) (Dhandapani et al. 1993), *Mamestra brassicae* L. (Vasconcelos 1996), *S. frugiperda* (Chapman et al. 1999b), granulovirus in *Plodia interpunctella* (Hübner) (Boots 1998), and an iridescent virus of *S. frugiperda* (Williams and Hernández 2006). In the current study, cannibalism rates increased when healthy and infected larvae were enclosed together, compared with those reared in separate groups. In-

deed, the highest incidence of cannibalism was observed in the high-density treatment involving 50% healthy and 50% infected fifth instars. In line with our findings, the patterns of transmission of lethal NPV disease in small boxes containing a majority of healthy *M. brassicae* larvae depended on the stages present and to a lesser extent on host density (Vasconcelos et al. 2002).

Viral infection seems to alter the risk of injury in interactions with conspecifics, because infected larvae may become less vigorous and therefore less capable of defending themselves than uninfected larvae, and hence more likely to become the victims of cannibals (Poprawski and Yule 1990, Boots 1998). The sluggish responses of infected insects probably result from major changes in the metabolic activity of infected insects (Thompson and Sikorowski 1981). Larval density also has been observed to have a significant influence on susceptibility to virus infection in *Spodoptera exempta* (Walker); insects reared at a high density were considerably more resistant to NPV disease than those reared in isolation (Reeson et al. 1998).

This study further underlines the findings of Lasa et al. (2007) who reported approximately three-fold increases in the production of OBs per larva in individually reared *S. exigua* sixth instars that had been treated with methoprene or fenoxycarb in the previous instar. In the current study, we observed that rearing density had a significant effect on OB production in fenoxycarb treated insects with increases of between 4.8- and 5.6-fold over fifth instars reared at high and low densities, respectively. This means that JHA technology could potentially be applied to baculovirus production in this and many other lepidopteran species. In the case of *S. exigua*, this technology had no adverse effects on the insecticidal properties of SeMNPV OBs or the prevalence of cannibalism. JHA technology is therefore likely to be compatible with mass production systems that employ practices involving gregarious rearing of infected insects.

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