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Increased plasma selenium levels correlate with elevated resistance of *Heliothis virescens* larvae against baculovirus infection

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Abstract

We reported that dietary selenium (Se) impacted the growth and development of *Trichoplusia ni* reared for many generations on diet containing extremely low levels of Se. Larvae had an elevated resistance to *per os* infection with a baculovirus. In this study, we examine how dietary Se (in the form of selenite) affects the growth, development, and Se content of *Heliothis virescens* that have been laboratory reared for less than two years. Larvae fed a commercial tobacco budworm diet supplemented with greater than 20 ppm Se grew at a slower rate than insects fed lower levels of Se and had an increase in the amount of Se sequestered in pupae. Larvae fed diets containing from 10–60 ppm Se exhibited elevated plasma concentrations of the micronutrient and increased plasma virucidal activity against *Helicoverpa zea* single nucleopolyhedrovirus (HzSNPV). Larvae reared on diet supplemented with 10 or 60 ppm Se until the onset of the penultimate instar were then infected *per os* or by injection with increasing concentrations of the baculovirus *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV). Larvae fed dietary Se and infected with occluded virus *per os* displayed a significantly lower mortality compared with infected larvae not fed Se. Our results suggest that dietary Se levels are directly correlated with plasma Se levels, and that plasma Se levels are in turn correlated with baculovirus resistance.

Keywords: *Heliothis virescens*; Selenium; Biological control; Resistance; Nutritional immunology; *Autographa californica* multiple nucleopolyhedrovirus; *Helicoverpa zea* single nucleopolyhedrovirus

1. Introduction

The micronutrient selenium (Se) plays a vital role in the resistance of vertebrates against viral infection (Beck et al., 2004). Se is a cofactor required for the activity of a number of selenoenzymes involved in the stress response, and the maintenance of high tissue antioxidant levels, which may contribute to a more robust antimicrobial and antiviral defense (Beck et al., 2004). We documented the possibility that Se may impact the efficacy of microbial biological control agents by tracking the mortality of Se-supplemented cabbage loopers, *Trichoplusia ni*, to the baculovirus *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) (Popham et al., 2005). We reported that the presence of Se in larval tissues during viral infection lowers the susceptibility of larvae, particularly in initial mortality. On the basis of this information we hypothesized that dietary Se supplementation would elevate tissue Se concentrations. We further hypothesized that elevated tissue Se concentrations would correlate with increased resistance against a baculovirus challenge. Here we report on experiments designed to test both hypotheses.

2. Materials and methods

2.1. Insects and insect diets

Heliothis virescens eggs were received from the North Carolina State University Department of Entomology Insectary. The insectary colony was established from field

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insects in July of 2002. Larvae were reared individually on an artificial wheat germ based diet (Catalog # F9781B, Bio-Serve, Frenchtown, NJ) under a photoperiod of 14 h:10 h (L:D) at 55% relative humidity at 28 °C (Popham et al., 2005; Shelby and Popham, 2006). Diets were supplemented with Se in the form of Na_2SeO_3 at 10, 20, 60, and 125 ppm. Diet without added Se was considered to be 0 ppm Se. Pupation, adult emergence and mortality data were collected daily on insects reared on increasing levels of Se. Midpoint pupation and emergence times were determined by the ViStat 2.1 program (Hughes et al., 1986).

2.2. Insect cells and virus

Two Cell lines, an *H. zea* cell line (*Hz*AM-1) and a *Spodoptera frugiperda* cell line (*Sf*21), were maintained as monolayers at 28 °C in Excel 401 medium (JRH Biosciences, Lenexa, KS) supplemented with 10% fetal bovine serum (Integen Co., Purchase, NY). Wild-type *H. zea* single nucleopolyhedrovirus (*Hz*SNPV) isolate was used and amplified in *Hz*AM1 cells for the virucidal assay (Popham et al., 2004). For larval bioassays, the L1 variant of AcMNPV was used and budded virus was amplified and titered in Sf21 for injection assays (O'Reilly et al., 1992).

2.3. Selenium content determination

Individual pupae, plasma samples, or small portions of diet were frozen, placed in pre-tared vials, oven dried at 65 °C and the dry masses calculated. Se determinations were performed by the University of Missouri Research Reactor by instrumental neutron activation analysis using a modification of the method described in (McKown and Morris, 1978). Se concentrations are expressed as ppm (μg Se/gm dry mass) and are presented as standard Tukey box plots showing the mean, median, and the 5th/95th percentile ranges (SigmaPlot 8.0, SPSS, Inc. Chicago, IL).

2.4. Plasma in vitro virucidal assay

Hemolymph was collected and virucidal activity in larval *H. virescens* plasma was quantitated by endpoint dilution assay as detailed (Popham et al., 2004). In short, hemolymph from early fifth instar larvae was collected directly into a chilled 1.5 ml microcentrifuge tube containing ice cold, sterile phosphate buffered saline (PBS) (50 mM $NaHPO_4$, pH 6.8). Hemolymph was adjusted to a final dilution of 1:10 by addition of cold PBS after which hemocytes were removed by microcentrifugation at 8000 rpm for three minutes. The plasma supernatant was sterilized by centrifugation at 6000 rpm for three minutes through a 0.65 μm Millipore Ultrafree™-MC centrifugal filter (Millipore, Inc., Bedford, MA). *H. virescens* plasma dilutions were combined with *Hz*SNPV at a ratio of 3:1 (v/v), gently mixed and allowed to incubate at 20 °C for 1 h. PBS was used as a control in the absence of plasma. Viral titers of these incubations were determined by end-point dilution assay (Popham et al., 2004). *Hz*AM1 cells were seeded at 5×10^4 cells ml^{-1} in 96-well plates (BD Falcon, Franklin Lakes, NJ) and allowed to attach for 1 h. The cells were infected with dilutions of virus/plasma or virus/PBS at dilutions of 10^{-1} to 10^{-6} and plates were incubated for 1 week at 28 °C. The plate wells were then scored positive, if polyhedra were visible within two or more cells, or negative for viral infection, and the results were used to calculate the viral titer as the tissue culture infectious dose per ml (TCID50 ml^{-1}) of inoculum. When indicated, statistical comparisons were done using the SigmaStat program (SPSS Inc., Chicago, IL).

2.5. Bioassays

Larvae fed diet with different levels of Se were challenged either *per os* or by injection with varying concentrations of AcMNPV at the onset of the fourth instar. For *per os* bioassays, *H. virescens* larvae were fed polyhedra isolated and sucrose gradient purified. Larvae were infected by the droplet feeding method (Popham et al., 2005) with doses ranging from 1×10^3 to 1×10^7 polyhedra/ml of AcMNPV and placed in individual cups with diet. Injection bioassays were performed by injecting larvae in a proleg with 2 μl of budded virus diluted to a treatment range of 0.0127 to 12.7 pfu/μl (Lapointe et al., 2004). Injections were done manually using a 5 μl Hamilton Syringe with a fixed 26 gauge needle and a beveled noncoring needle point. Larvae were monitored two or three times daily for death for 10 days and the times the larvae were monitored was recorded. *Per os* and injection bioassays each contained five doses of virus with 30 larvae/dose. *Per os* assays were repeated four times and injection assays twice.

2.6. Statistical analysis of bioassays

With multiple factors and the potential of interactions among these factors, something other than LC_{50} is needed for the analysis of bioassay data. Use of a Generalized Linear Model (Nelder and Wedderburn, 1972) allows us to deal with the complexity of a multi-factor experimental design with a binary response. In this paper, the probability of death was modeled with Generalized Estimating Equations (GEE) (Liang and Zeger, 1986), which allows an analysis that deals with the non-independence of observations through time.

Graphic examination of the data showed there were few deaths before 120 h and the number of deaths remained fairly constant at more than 80% after 192 h. Because this consistency of no deaths or almost all deaths masked the effects of viral dose, Se dose and time only data from 120 to 192 h were included in the analyses.

In this study, there were two factorial experiments (*per os* and injected) with the factors: (1) Se dose level, (2) viral dose level, and (3) time, in each experiment. Analytical tools exist which can take into consideration that a factorial experiment was performed and also model the suspected