# Effects of *Aspergillus niger* Contamination on Biological Fitness of *Lygus hesperus* (Heteroptera: Miridae)[1]

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J. Entomol. Sci. 37(4): 338-344 (October 2002)

**Abstract** The effect of *Aspergillus niger* (USDA, ARS Robert T. Gast Rearing Laboratory, Mississippi State, MS isolate) contaminated artificial diet on *Lygus hesperus* Knight (Heteroptera: Miridae) is described. A standard number of *A. niger* spores was inoculated into an artificial diet used to rear *L. hesperus*, and the effect on biological fitness of the insect was measured. Biological fitness was defined as total number of surviving adults, mean biomass (dry weight) accumulated per cage over the total test period, egg production, time to adult emergence, and time to the beginning of egg laying. These measurements were all significantly different for insects reared on the inoculated diet from those reared on the control diet. The insects reared on the diet inoculated with *A. niger* showed a high mortality of nymphs, a decrease in mean biomass, delayed development time, and a decrease in egg production. This study demonstrates the devastating effect *A. niger* diet contamination can have on a laboratory colony of *L. hesperus* and emphasizes the need for constant vigilance and adherence to strict sanitation methods in an insect rearing facility.

**Key Words** Plant bugs, mass rearing, artificial diet, *Aspergillus niger*

Controlling mold is one of the most challenging problems for an insect mass rearing facility (Funke 1983). The most commonly encountered species of mold are those of *Aspergillus,* particularly *Aspergillus niger* van Tieghem (Clark et al. 1961, Ouye 1962, Singh and Bucher 1971, Gifawesen et al. 1975). A high quality artificial diet that can be used to rear large numbers of the targeted insect species is a major component of a successful rearing program (Nordlund and Greenburg 1994, Nordlund 1996, Cohen 2000a, b). It is difficult, however, to rear high-quality insects in an insectary that has a high incidence of microbial diet contamination. These contaminants can have serious adverse effects on the development and health of the reared insects. Many different species of microorganisms can utilize the growth media of insect artificial diets as they are usually nutrient-rich, and the insects are usually grown in conditions of high humidity and temperature that are also ideal for microbial growth.

*Aspergillus niger* contamination in insectaries has been associated with high mortality of young insects, a prolonged development time, and smaller than normal larvae and adults (Howell 1971, Singh and Bucher 1971, Sikorowski et al. 1980). The ability of the microorganism to colonize the insect diet and the stage and age of the insect

[1]Received 19 December 2001; accepted for publication 31 March 2002.

at the time of contamination influence the effect of the microbe on the insect. Larvae of the codling moth, *Laspeyresia pomonella* L., exposed to diet contaminated with *A. niger* either abandoned the affected areas of the diet, stopped feeding and died from starvation, or crawled through the contaminated diet, then became covered with fungal conidia and died (Howell 1971). Artificial diet contamination by *A. niger* interfered with larval development in *Agria affinis* auct. nec Fallén, and all insects died within 10 d without reaching the third stadium (Singh and Bucher 1971). Sikorowski et al. (1980) observed that if *A. niger* was present in 1- to 2-day-old colonies of boll weevils, *Anthonomus grandis grandis* Boheman, there was 100% larval mortality, but if colonies were infected in late larval stage, the number of emerging adults was not noticeably affected (Sikorowski et al. 1980). This demonstrated how *A. niger* contamination and its effects may be difficult to detect and manage in the insectary. A chronic contamination of older insects may go unnoticed until it is too late and the microbe has wiped out the next generation.

Western tarnished plant bugs, *L. hesperus,* are major pests in many cropping systems in North America. They are reared at the Robert T. Gast Rearing Laboratory to provide material for entomologists to conduct research on biological control, physiology and behavior of these insects. The purpose of this study was to determine the effect of *A. niger* contamination on *L. hesperus.* The hypothesis was that exposure to the insect by this microbe would have a negative impact on the development and fecundity of *L. hesperus.*

## Materials and Methods

**Insects.** *Lygus hesperus* used in these studies were obtained from a colony from Biotactics, Inc. (Riverside, CA), colonized on the "C diet" at the Robert T. Gast Rearing Laboratory for 1.5 yrs (Cohen, unpubl. data), and on the new NI diet (Cohen 2000a) for the 29 generations (as of January 2001) previous to this experiment. The NI diet used in these experiments was a combination of whole eggs and egg yolks, soy flour, wheat germ, lima bean meal, yeast, and vitamins (Cohen 2000a). Voucher specimens of *L. hesperus* were placed in the Mississippi State University Entomology Museum (Mississippi State, MS).

**Experimental protocol.** Cages used for all life stages were Rubbermaid® 1.7-L Servin' Saver™ (Wooster, OH) rectangular plastic storage boxes. Cages were topped with organdy cloth (0.4-mm mesh) held tight by the box's snap-on top that had 15.7 × 26.5 cm opening cut into it. The organdy cloth was changed to a 1.0-mm mesh fiberglass screen when the nymphs were large enough (approximately third instar) to be contained by the larger mesh. Growth chamber (Powers Scientific model DS33SD, Pipersville, PA) conditions were a light-dark cycle of 16:8 h, temperature of 27°C (± 1.0°C), and relative humidity of 60% (± 2%). Cages were placed on wire racks that allowed air circulation and light to reach each cage. A system for feeding in Parafilm® (Pechiney Plastic Packaging, Menasha, WI) packets and oviposition in Parafilm packets filled with gel has been developed (Patana 1982), and was used in this study. The test colony was treated according to the recommendations of previous researchers (Debolt and Patana 1985, Cohen 2000a), except for the modifications specified here. Original egg packets used to start the test were placed intact into cages rather than being separated from the gel. To reduce cannibalism, egg packets were placed inside cages with shredded paper (0.6 × 28.4 cm) rather than loosely wadded paper towels. Feeding packets and gel packets were made from 20.8 × 10 cm strips of Parafilm,

folded and sealed with a heat sealer (Deni Freshlock Turbo II, Keystone Manufacturing, Co., Buffalo, NY) along two sides to create a 10.4 × 10 cm packet with an open top for filling. The first feeding packets provided to newly-eclosed nymphs were stretched by hand to facilitate feeding, and one stretched packet was placed inside the cage as well as on top of the organdy. Feeding packets on the tops of the cages were replaced every 48 h and stretched until the nymphs were second instars (approximately post oviposition day 9). The feeding packets inside the cages and the original egg packets were removed between days 10 and 12, and the organdy was replaced by screening, as nymphal size allowed. Once adults emerged, a 2% Gelcarin® (FMC-Food Ingredients Division, Rockland, ME) gel packet was placed on top of the cage for oviposition and changed daily. Cohen (2000a) indicated that a higher percentage gel than formerly used (Debolt and Patana 1985) increased egg hatch.

**Experimental contamination of the artificial diet.** The control and *A. niger* inoculated diets consisted of NI ingredients prepared as previously described (Cohen 2000a) except the formalin and propionic acid were omitted. All diet preparations were conducted in a laminar flow hood or biological safety cabinet as appropriate. The pH of the diets was 5.5 ± 0.2. Approximately 20 ml of diet was dispensed into each feeding packet, and the tops were heat sealed.

The *A. niger* was isolated at the Robert T. Gast Rearing Laboratory and, therefore, represents a microorganism that is a potential contaminant of artificial diet at this laboratory. *Aspergillus niger* conidia were harvested after 5 to 8 d of growth at 27°C on Sabouraud's dextrose agar (SDA) (Difco, BD Diagnostics Systems, Sparks, MD). They were harvested by lightly scraping the surface of the mold with a sterile cotton swab dampened in sterile water. The black conidia were rinsed off the swab into a sterile tube containing 2 ml of sterile water. The diet packs for the fungal challenge were inoculated with a working solution of *A. niger* (2000 spores in 0.3 ml) via syringe just prior to heat sealing the tops. This resulted in a final dilution of 100 spores per ml of diet. This inoculum was chosen because it is within the range likely to occur when insect diets are contaminated by chance (Singh and Bucher 1971). *Aspergillus niger* growth was visually evident in the experimentally inoculated feeding packets. When the feeding packets were changed after a 48 h feeding time, the packet contents were streaked on SDA plates and grown at 27°C to confirm *A. niger* growth from inoculated feeding packets, and no growth from control feeding packets.

**Experimental design.** To begin all tests, rearing units consisting of individual cages, described above, were set up and infested with a Parafilm packet containing 200 eggs. All test eggs for each replication were collected within a 2-h oviposition period. The following parameters were measured for each treatment group: (1) total number of surviving adult; (2) the biomass (dry weight) accumulated per cage over the total treatment period, including adults, deceased nymphs, and exuviae; (3) time to adult emergence; (4) time to the beginning of oviposition; and (5) number of eggs produced by each cage of adults per day for 5 d. The experiment was terminated 26 d after hatch of the test insects (Debolt and Patana 1985). Surviving adults were collected and killed by freezing at −20°C for 4 to 6 h. Dead nymphs and exuviae also were collected and frozen. Dry weights were obtained after drying both collections at 70°C for 48 h. The collections were combined for each cage to determine a total biomass. Hatch rates from the original egg packs used to begin the experiment and from the F1 egg packs were calculated based on counts of microscopically observed opened opercula and empty eggs. Three replications were performed. Blocks consisted of replication in time. Data were analyzed using analysis of variance (ANOVA),

and treatment means were compared by Fisher protected least significant difference (LSD) (SAS Institute, 2000).

## Results and Discussion

All measurements for biological fitness (total number of surviving adults, mean biomass [dry weight] accumulated per cage over the total treatment period, egg production, time to adult emergence, and time to the beginning of egg laying) were significantly affected in the insects reared on the diet experimentally inoculated with *A. niger* (Table 1). Of the original 200 eggs, the percentage of adults surviving until the experiment was terminated was 75% on control diet and 53% on contaminated diet. The hatch rates from the original egg packs were between 90 and 95% for all cages in both diet treatments. Few adults died in the tests, an average of 2 per cage for the control diet and 3 per cage for the inoculated diet. Most insect death occurred in the nymphal stage for both diets. The mean biomass production per cage for insects reared on the inoculated diet was 70% that of insects on the control diet. Development was delayed in the insects reared on the inoculated diet as evidenced by the significant increase in the time to adult emergence (2.4 d) and time to the beginning of oviposition (2.6 d). Egg production was dramatically decreased in insects reared on the inoculated diet as shown by the egg per female per day calculation in Table 1, and also by the plot of the number of eggs per cage per day for the 5 d eggs were collected in Figure 1. The mean total number of eggs produced for each diet for the 5-d period was 10,774 for the control diet and 2,527 for the contaminated diet, a decrease of 76.5% due to *A. niger*. It is interesting to note that even though the number of eggs per cage per day was significantly decreased each day for insects reared on the inoculated diet, the day of peak oviposition was the same (day 4) for each treatment. The F1 hatch rate for all of the experimental insects was between 87 and 95% and was not affected by diet treatment.

The major purpose of a mass rearing program is the efficient production of high quality insects (Cohen 2000a). Biological fitness, as defined in this research, has major impact on the quality of the product being raised and on the cost effectiveness of the program. Delays in insect development, decreases in fecundity and biomass accumulation, and an increased mortality of nymphs are all factors indicating problems in a rearing program.

**Table 1. Effects of *Aspergillus niger* on biological fitness of *Lygus hesperus***

| Diet | Total number of surviving adults | Total biomass (dry weight in mg) | Eggs per female per day | Days to adult emergence | Days to oviposition |
|---|---|---|---|---|---|
| Control | 150.3 ± 3.9 a | 772.9 ± 7.0 a | 28.9 ± 0.8 a | 15.3 ± 0.3 a | 19.7 ± 0.3 a |
| Inoculated with *A. niger* | 106.7 ± 3.2 b | 546.5 ± 11.9 b | 9.1 ± 0.2 b | 17.7 ± 0.6 b | 22.3 ± 0.3 b |

Means ± SE within the same column followed by a different letter were significantly different (LSD test, $P < 0.01$, SAS Institute, Inc., software version 8.01, 2000).

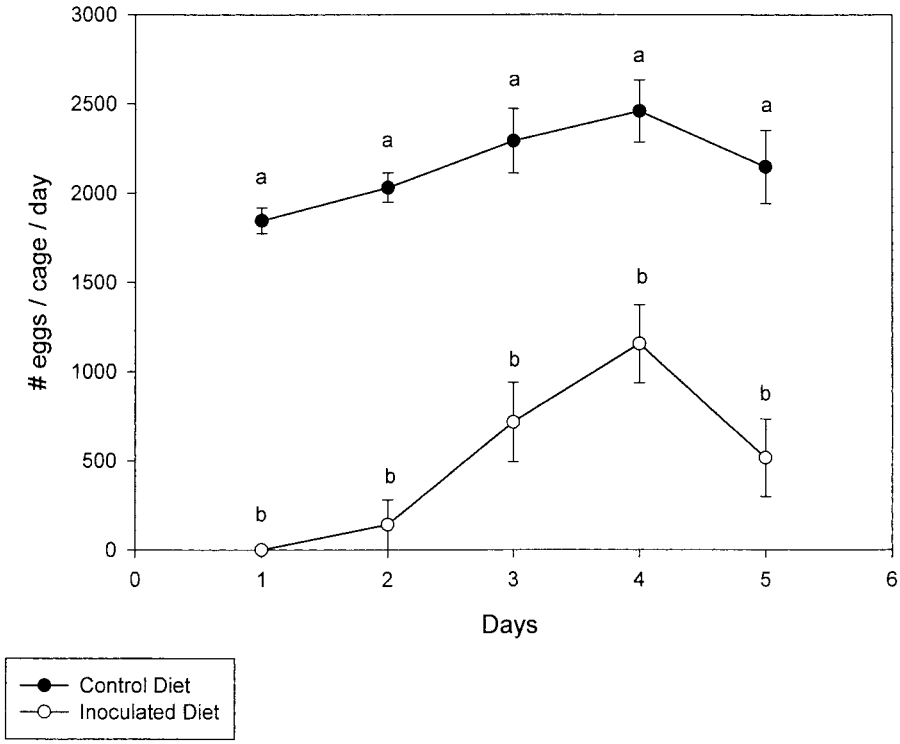


Fig. 1. Egg production by *Lygus hesperus* on control diet and diet inoculated with *Aspergillus niger*. Points represent the mean number of eggs for each diet on each day (± SE). In individual day treatment comparisons, data points with different letters were significantly different as indicated by Fisher protected LSD ($P < 0.01$), SAS Institute, Inc., software version 8.01, 2000.

This diet contamination study showed the highly toxic nature of *A. niger* to *L. hesperus*. Insects reared on diet contaminated with this microbe were significantly reduced in quality as defined by measurements of biological fitness. Spores of *A. niger* are airborne, thus all of the insects in the growth chamber were potentially exposed to spores released from the mouthparts of insects during feeding on the diet packs and subsequently detached and moved about the cage. The insects feeding on the inoculated diets, however, were much more severely affected than insects merely exposed to airborne spores. Other *Aspergillus* spp. such as *A. flaxus* produce toxins, some of which are insecticidal (Beard and Walton 1969, Toscano and Reeves 1973). Because of the rapid death of larvae in *A. niger* contaminated media, Singh and Bucher (1971) suspected some toxic action was at work, but did not elucidate the mechanism (Singh and Bucher 1971). *Aspergillus niger* produces oxalic acid and kojic acid abundantly, but these compounds have shown only a slight acute toxicity in rats (Ueno and Ueno 1978). Malformins produced by *A. niger* are more potent toxins, at least by intraperitoneal injection in mice (Kobbe et al. 1977). Pathologic signs

accompanying mouse fatality included dilatation with hemorrhage of the gastrointestinal tract, changes in the liver and kidney, and death occurred within 4 d of injection. Oral doses, however, failed to cause evidence of acute toxicity (Yoshizawa et al. 1975). Of course, these results from mammalian studies may not translate to the mode of action in insects. Clearly, more research is required to clarify the mechanism by which *A. niger* adversely affects *L. hesperus* and other insects on artificial diets.

Research on insects for biological control, physiological, or behavioral purposes depends on the availability of high quality insects produced by reliable insect rearing techniques (Davis and Guthrie 1992, Sikorowski and Lawrence 1994, Cohen 2001). Microbial contamination in the insectary, as demonstrated here, can lead to a wide fluctuation in insect quality and would be expected to cause considerable increases in production expenses and employee work loads. A fluctuation in insect quality can also lead to a loss of confidence in the product by the client, because the results from laboratory experiments using inferior insects are often inaccurate. This study emphasizes the necessity of strict sanitation in the insectary.

## Acknowledgments

I thank Lois Connington for assistance in conducting the bioassays, Gay McCain and Brenda Woods for technical support in rearing the *L. hesperus* colony from which research insects were obtained, Debbie Boykin for statistical advisement, and Frank M. Davis (Mississippi State University) and Michael R. McLaughlin (USDA, ARS, Mississippi State, MS) for reviews of an earlier draft of this paper. This manuscript is Mississippi Agriculture and Forestry Experiment Station research article No. (J10025).

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