Effect of DNA Gyrase Inhibitors in the NI Diet on Biological Fitness of the Western Tarnished Plant Bug (Heteroptera: Miridae)¹

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Abstract Addition of 3 DNA-gyrase inhibitors (i.e., novobiocin, nalidixic acid and oxolinic acid) individually to the NI (New and Improved) oligidic diet for plant bugs was investigated to assess their effect on the biological fitness of *Lygus hesperus* Knight (Heteroptera: Miridae). Biological fitness was measured by the number, biomass, sex ratio, daily egg count per female, and the egg hatch rate. All measures of biological fitness were not significantly different among the controls and the 3 inhibitors, which demonstrated that the inhibitors can effectively replace present antibiotics for a less expensive diet. Females reared on the NI diet with novobiocin and oxolinic acid showed increased fecundity in comparison with the diet with nalidixic acid, but the types of inhibitors had no effect on egg hatch rate. Furthermore, the egg hatch rate during the first 12 d after oviposition produced the most viable eggs, with the highest hatch rate between the 2nd and 9th days after oviposition commenced, which is comparable to the current egg-collection period used for the *L. hesperus* colony. These inhibitors could replace the antibiotics currently used in the NI diet and reduce the cost for diet preservation without sacrificing the quality of the insects in the mass-rearing facility.

Key Wordsnovobiocin, nalidixic acid, oxolinic acid, mass rearing, quality control, shelf life

The western tarnished plant bug, *Lygus hesperus* Knight (Heteroptera: Miridae), is an important economic pest worldwide, because its wide host range includes a variety of important seed and fruit crops (Wheeler 2001). A mass-rearing system for this species on artificial diet was developed in the 1980s by Debolt (1982), Debolt and Patana (1985) and Patana (1982), and improved for fungal control by Cohen (2000) and Alverson and Cohen (2002). The mass-reared insects have served research projects ranging from physiological and behavioral, to biological control studies of the pest. In addition, this prolific pest has been used as a host for mass rearing of biological control agents, such as the mymarid wasp, *Anaphes* spp.

In general, insect diets are maintained at rearing temperatures conducive to microbial growth. Therefore, it is not surprising that microbial contamination of artificial diets and the presence of insect pathogens have periodically interrupted the mass rearing systems for a number species of insects (Soares 1992, Sikorowski and

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Lawrence 1994, 1997, Sikorowski et al. 2001). The mass rearing system of *L. hesperus* is no exception with the artificial diet plagued periodically by bacterial and/or fungal contaminations in the rearing facilities. The mold *Aspergillus niger* van Tieghem is a major contaminant in insect-rearing facilities and requires effective control to produce quality insects. Alverson and Cohen (2002) and Alverson (2003) examined and reported the use of lipophilic acids to reduce *A. niger* contamination in the mass rearing of *Lygus* spp. Bacterial contamination also has been reported in the new and improved (NI) oligidic diet, and research on the reduction of bacterial contamination in the NI diet was needed.

The routine use of antibiotics to prevent bacterial contamination in insect mass rearing has two disadvantages: significantly increased rearing costs and effects of antibiotics in artificial insect diets on the mass-reared insects. Thus, research on novel antibiotic substitutes is needed. Büyükgüzel (2001a, b) reported that the addition of DNA-gyrase inhibitors (as known as bactericides) in the artificial diet of *Pimpla turionellae* L. (Hymenoptera: Ichneumonidae) had positive effects on the biological fitness of the wasps. Objectives of the present experiment were to determine the effect of three DNA-gyrase inhibitors (i.e., novobiocin, nalidixic acid, and oxolinic acid) on *L. hesperus* biological fitness.

**Materials and Methods**

**Insects.** *Lygus hesperus* used in this study were derived from a colony established by Biotactics (Riverside, CA). The colony had been maintained in the Biological Control and Mass Rearing Research Unit on the NI diet for over 5 yrs (Alverson 2003) before conducting this experiment. The experiment was conducted in the Gast Rearing Facility, Mississippi State, MS, at 25°C, 50% RH, and a photoperiod of 16:8 (L:D). The insect colony also was maintained in the same facility under the same conditions.

**Inhibitor treatments in artificial diet.** The NI diet was made following the methods described by Cohen (2000). The DNA-gyrase inhibitors used in the experiment were novobiocin (sodium salt, 90%) (ICN Biomedicals, Inc., Costa Mesa, CA), nalidixic acid (99%) (Sigma Chemicals, St. Louis, MO) and oxolinic acid (99%) (Sigma Chemicals, St. Louis, MO). A volume of 1.5 ml of desired concentrations (i.e., 5, 10, 15, and 20 mg) of each inhibitor was prepared in autoclaved Millipore water. The 1.5 ml inhibitor solution was blended into an aliquot (100 g) of the NI diet. The blended diet was allocated equally into four diet packets that were made of Parafilm “M” laboratory film (American National Can™, Greenwich, CT). The two controls were normal NI diet, and the NI diet minus two antibiotics (i.e., chlortetracycline and streptomycin sulfate) in the recipe plus 1.5 ml of autoclaved Millipore™ water. The purpose of using these two controls was to compare the bactericidal property of the inhibitors with the antibiotics that had already been included in the diet and to exclude any confounding or synergetic effects among the inhibitors and the antibiotics in the NI diet. The diet packets were prepared once a week and stored in a refrigerator (4°C) until use.

**Effect of the inhibitors on insect biology.** The experiment was initiated using newly-collected eggs (exactly 200 eggs oviposited within 1 h) in a portion of a gel packet for each of the 18 treatments. Each of the trials lasted a month, and was terminated after examination for egg viability (i.e., hatch rate) of the next generation. To prevent the negative effect of bacterial spoilage on insect development, all experimental diet packets were changed every 48 h throughout the experiment. The
parameters used to assess the effect of the inhibitors on insect development, as described by Büyükgüzel (2001b) and Alverson and Cohen (2002), included sex ratio, percentage of females, and dry weight (biomass) of the experimental insects. The number of adults (live and dead), the number of females (live and dead), and the biomass of insects from each treatment were recorded and compared at the end of the experiment. After the number and sexes of the adults had been recorded for each treatment, the insects were killed by freezing, and the dead insects were dried in an oven with 70°C for 48 h, as described by Alverson and Cohen (2002). The fecundity (or the number of eggs per female) was then calculated for each treatment.

Eggs were collected using 2% agar gel packets during the adult stage. Gel packets were replaced every 24 h for the first 12 d of oviposition during the adult stage. The number of eggs was counted under a stereo microscope. A daily egg count per female (ranging from 3.5-33.6) was calculated by counting the total number of eggs in a gel packet divided by the number of females in a particular rearing cage, which was recorded at the end of the experiment. The gel packets with eggs were incubated individually for a week in Petri dishes until no nymphs could be collected. When the eggs started to hatch, nymphs were removed from the Petri dishes every 12 or 24 h to minimize nymphaal predation on the unhatched eggs. When no nymphs hatched, the unhatched eggs on a gel packet were counted under a stereo microscope. The percentage of the viable eggs (or egg hatch rate) was calculated by subtracting the number of unhatched eggs from the total number of eggs during the sampling period (1-12 d after initial oviposition).

The “preservative effect” of the inhibitors on the NI diet packets was examined for 7 d in the rearing room under the conditions described previously, although the diet packets for insect rearing were replaced every 48 h. The gas-producing spoilage of the diet packets was assessed using visual evaluation of the diet packet inflation caused by gas production inside the packets. Two replications were conducted for this examination at the same time that biological fitness was assessed.

Experimental design and data analysis. This experiment was a split plot design with two trials considered as the main plot. The six treatments for each of the three inhibitors were subplots, including the two controls (NI diet, and the NI diet minus antibiotics) and the 4 concentrations 5, 10, 15 and 20 mg per 100 g of the NI diet. Data were analyzed using the PROC MIXED procedure of the SAS software (SAS Institute 2003). Means were separated using Fisher’s protected LSD test ($\alpha = 0.05$).

Results

Effect of the inhibitors on L. hesperus fecundity. Because the 4 levels (5, 10, 15 and 20 mg per ml) of the 3 inhibitors had no effect ($P$ values $> 0.05$) on insect development, the developmental data were pooled for further analyses. The pooled data showed that the five diet treatments (i.e., the NI diet, NI diet without antibiotics, and the NI diet with the three inhibitors, respectively) significantly affected total number of eggs deposited per female during the first 12 d of the oviposition period ($F = 3.44; \text{df} = 4, 26; \ P = 0.022$). When compared with the 2 controls of the experiment (i.e., the NI diet and the NI diet without antibiotics), the addition of inhibitors showed no negative effect on developmental indices of L. hesperus, including number of adults, female percentage, insect biomass, female fecundity, and egg hatch rate for the following generation. The addition of either novobiocin or oxolinic acid in the NI diet significantly increased the fecundity of L. hesperus when compared with the diet
to which nalidixic acid was added (Fig. 1). However, the effect of nalidixic acid on female fecundity was not different from the two NI-diet controls (i.e., NI diet and NI diet without antibiotics) (Fig. 1). Furthermore, the overall egg hatch rate was not significantly different ($F = 1.02; df = 4, 26; P = 0.4123$), ranging between 78 and 81% among the five treatments (Fig. 1).

**Influence of the inhibitors on daily egg count per female and egg hatch rate.**

The pooled data showed that the 5 diet treatments did not affect ($F = 1.33; df = 4, 4; P = 0.3955$) the daily egg counts per female during the first 12 d of the oviposition period examined, nor did the treatment by sampling time interaction affect the daily egg counts per female ($F = 1.05; df = 44, 362; P = 0.3858$). However, the sampling time significantly ($F = 42.84; df = 11, 362; P = 0.0001$) affected daily egg counts per female (Fig. 2). The daily egg counts (number of eggs deposited per female per day) increased by 20 eggs per female between the 6th and 12th d compared with the 1st and the 5th d. Similarly, the egg hatch rate during the 12-d oviposition period was affected significantly by sampling time ($F = 5.75; df = 11, 352; P = 0.0001$), but not affected by either diet treatments ($F = 0.66; df = 4, 4; P = 0.6523$) or by treatment by

![Graph showing the effect of dietary treatments on female fecundity and egg hatch rate.](image)

**Fig. 1.** Effect of five NI diet treatments on female fecundity and egg hatch rate of *L. hesperus* (n = 12). Bars with different letters (a-b) in the bar graph are significantly different (LSD, $P < 0.05$), whereas the line graph showed that the egg hatch rate was not significantly different among the five diet treatments.
Fig. 2. Effect of five NI diet treatments on *L. hesperus* egg counts per female per day during the first 12-d of the oviposition period.
sampling time interaction ($F = 0.57; \text{df} = 44, 352; P = 0.9857$). Because egg hatch rate was not affected, the data from the 4 levels of each inhibitor were pooled to assess the effect of sampling time on daily egg counts. During the 12-d oviposition period, the egg hatch rate among the 3 inhibitor treatments was greater (over 80%) between the 3rd and 9th day than for the remainder of the oviposition time (i.e., 1st, 2nd, 10th, 11th, and 12th day) (Fig. 3A-E).

We also observed that the addition of the inhibitors as low as 5 mg per 100 g diet could effectively prevent gas-producing spoilage when compared with the negative controls (the NI diet minus antibiotics) of the experiment. The diet packets for the negative control (i.e., the NI diet without any antibiotics) were no longer useful after being maintained at 25°C for 7 d; the diet packets became inflated by the gas produced inside the packets. However, the positive control (i.e., the NI diet) and three inhibitors (with $\leq$5 mg per 100 g concentration) showed no gas production, and no signs of spoilage in the diet packets after the 7-d experimental period.

**Discussion**

Given that the antibacterial property of DNA-gyrase inhibitors has been well documented and the positive effect on parasitoid wasps is known (Buyukguzel 2001a, b), the present study is one of the first attempts to assess the influence of DNA-gyrase inhibitors on biological fitness of *L. hesperus*, a piercing-sucking insect, for the purpose of mass rearing. Typically, insect mass-rearing facilities are being held under optimal conditions for insect development, which usually coincides with optimal microbial development conditions. Microbial contamination would accelerate the chemical reactions that lead to deterioration of nutritional values of the diets by reactive-oxygen species (Cohen 2003). Cohen and Crittenden (2004) further described that both cryptic and added antioxidants in artificial diets are critical in protecting the diets from oxidative deterioration. Like human food, diets for insect development should consider techniques to prevent microbial contamination and preserve antioxidants. Thus, when compared with the negative controls in the experiment, prevention of gas-producing spoilage in the NI diet with the DNA gyrase inhibitors will require further examination. The lack of gas production in the inhibitor-treated diet packets could be the result of 2 mechanisms. Either the inhibitors prevented gas-producing bacterial growth, or the inhibitors preserved antioxidants in the NI diet, thus preventing the gas-producing chemical deterioration (e.g., oxidation) of the diet ingredients as described by Cohen and Crittenden (2004). Additional studies of this preservation mechanism will allow us to improve the diet quality, as well as to preserve the diet's nutritional value.

The high fecundity of the females also could result from enhancement of bacterial symbionts in *L. hesperus*. Douglas (1998), Moran and Telang (1998), and a number of other researchers have demonstrated that plant sap-feeding insects rely solely on their obligate symbionts (e.g., *Bruchnera* spp.) to provide all essential nutrients. Asymbiotic insects could not develop properly because plant sap provides only limited nutrients. Bacteria of the genus *Wolbachia* contribute to the reproductive success of arthropods (Bourtzis and O'Neill 1998). The inhibitors might have altered the bacterial or microbial flora in insect alimentary tract and/or reproductive systems, leading to the change in daily egg count per female, and variation of egg hatch rate during the 12 d period. Thus, the association between *Lygus* and bacterial gut flora may be more complex than previously reported. The mechanisms underlining the positive effect of
Fig. 3. Temporal change in egg hatch rate of *L. hesperus* reared on five NI diet treatments (A-E) in the first 12 d of oviposition period. The bars with different letters (a-d) denote that they are significantly different (LSD, *P* < 0.05).
novobiocin and oxolinic acid, but not nalidixic acid, on daily egg count of *L. hesperus* will require further investigation.

Because different inhibitor levels (i.e., 5, 10, 15, and 20) showed no effect on insect fecundity in the present study, additional experiments will be necessary to determine the optimum level (in the range between 5 and 10 mg) of the inhibitors to further enhance the NI diet. These experiments would be required to reduce the overall cost for *L. hesperus* production. Büyükgüzel (2001a) had conducted similar experiments to assess the positive effect of DNA gyrase inhibitors on *P. turionellae*. In the first experiment, 5, 10, 15, and 20 mg inhibitors per 100 g of the diet were used. Based on preliminary results, the range of the inhibitors was narrowed into 1.5, 3.0, 4.5, and 6.0 mg per 100 ml diet for novobiocin, 1.0, 2.0, 3.0, and 4.0 for nalidixic acid, and 0.75, 1.50, 2.25, and 3.0 for oxolinic acid (Büyükgüzel 2001a). *Lygus hesperus* might have a similar dose response to various concentrations of the inhibitors like the parasitoid *P. turionellae*. Thus, after the present study demonstrated no negative effect of the three inhibitors on *L. hesperus* biological fitness in comparison with the control diets, future studies on dose response should provide the optimum concentration of the inhibitors in the NI diet to reduce the cost of mass rearing quality insects. The addition of the inhibitors effectively prevented the gas-producing spoilage of the NI diet for 7 d at 25°C, which served the same purpose as adding antibiotics to the diet. We found that the inhibitors could replace antibiotics used in the standard NI diet without adversely affecting the shelf life of the NI diet. Furthermore, the addition of either novobiocin or oxolinic acid to the NI diet increased female fecundity when compared with nalidixic acid. Although egg-hatch rate was not affected by the three inhibitors, the egg hatch rate was variable during the 12 d oviposition period. Between the 3rd and 9th d more than 80% of the eggs hatched. Either novobiocin or oxolinic acid could replace currently used antibiotics in the NI diet and reduce the cost for diet preservation without sacrificing the quality of insects produced in the mass-rearing facility.

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