Juvenile Coloration as a Predictor of Health in *Nezara viridula* (Heteroptera: Pentatomidae) Rearing[1]

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Abstract Field collections of early nymphs of *Nezara viridula* (L.) revealed that normal change in coloration pattern occurring between the fourth and fifth stadia do not occur in some individuals producing abnormally-colored fifth instars. To determine if abnormal coloration is an indicator of poor health, field-collected fifth instars were grouped in 4 coloration pattern groups: (1) Green, normal coloration pattern; (2) faded, displaying similar patterns as "green" but with paler colors; (3) intermediate black-green ; and (4) black, retaining fourth-instar coloration pattern. The fitness of nymphs within coloration pattern groups was measured by recording survival to adulthood and assessment of adult weight, fecundity, and egg viability. Adults originating from each nymphal coloration pattern were grouped to determine if the variability in nymphal coloration was hereditary. Survival from fifth instar to adult was significantly lower in nymphs displaying abnormal coloration patterns showing 21 and 12.5% survival in the black and faded groups, respectively, as compared with 76.2% in the normal green coloration group. Adults originating from nymphs of black coloration did not produce progeny, whereas adults originating from the faded coloration produced significantly less eggs with lower viability than adults originating from nymphs with normal coloration. However, adults originating from normally-colored nymphs produced progeny of abnormal coloration in similar frequency as adults originating from black and intermediate colored nymphs. Abnormal nymphal coloration can be used as an indicator of health and can aid in the selection of healthy individuals for culture.

Key words: southern green stink bug, survival, fecundity, rearing, nymphal coloration

The southern green stink bug, *Nezara viridula* (L.), is a world-wide polyphagous pest that damages economically-important crops such as soybeans, green beans, black-eyed peas, cotton, and peanuts (Hirose et al. 2006). Additionally, *N. viridula* transmits disease-causing organisms to citrus and cotton bolls (Mitchell 2004, Medrano et al. 2009, Shivas et al. 2005, Esquivel et al. 2010). Because of its economic importance as a cosmopolitan pest, the biology of this insect is a continuing area of research. Thus, this insect is reared in laboratories around the world on raw, shelled peanuts, fresh soy bean pods, and/or fresh bean pods (Jones 1985). Further, addition of Streptomycin sulfate to drinking water provided to first-instar nymphs resulted in beneficial effects to the subsequent colony (Hirose et al. 2006). The life cycle of *N. viridula* includes 5 nymph instars, most of which have a dorsal black and white dotted coloration pattern. Dramatic coloration changes normally occur between the fourth and fifth stadia. Coloration varies in the fourth stage but the majority that reach fifth instar present a green coloration

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without a significant difference on survival and weight due to diet (Brown 1984). Alternatively, Panizzi and Slansky (1991) reported that the weight and survival of nymphs and adults were impacted by changes in food sources, but immature coloration changes were not reported.

Nezara viridula is currently reared at USDA, ARS, National Biological Control Laboratory (Stoneville, MS) either on raw vegetables or on a recently developed-artificial diet (Rojas et al. unpubl). During the development of artificial diets, it was noted that when insects were fed suboptimal artificial diets, the proportion of fifth instars displaying fourth instar-like coloration patterns increased as well as their mortality rate. In some instances, these coloration changes failed to occur and an array of different intermediate coloration patterns were observed. The body color ranged from mostly green to black and combinations thereof. Based on these observations, our objectives were to (1) determine if the abnormal coloration patterns of fifth instar nymphs reflect a decline in colony health and vigor, and; (2) elucidate whether the abnormal coloration patterns in fifth instars persists to the next generation of fifth instars, in adults originated from normally colored fifth instar nymphs.

Materials and Methods

Nezara viridula nymphs and adults were collected from soybean using a sweep net during August-September of 2010 around the USDA ARS Mid South Area campus in Stoneville, MS. Collected specimens of *N. viridula* were positively identified in the laboratory, separated by stage of development, and 15 individuals of each stage (fourth and fifth instars, or adults) were temporarily transferred to plastic rectangular boxes (L 343 X W 203 X H 127 mm, Sterilite, Townsend, MA), with 4 screened windows (30 mm dia.) on the top. Food consisting of raw peanuts, fresh broccoli, and fresh green bean pods was refreshed every other day. Boxes were maintained in an environmentally- controlled room at 26 ± 2°C, 67± 5% RH, and 16:8 (L:D) h photoperiod (Hirose et al. 2006, Brewer and Jones 1985). Crumpled pieces of paper towels within the boxes provided oviposition media for the females. Eggs were daily collected and used to establish the *N. viridula* colony.

Rearing procedures of stock colony. To reduce food spoilage, adult *N. viridula* were reared using cages constructed from clear plastic boxes (L 320 X W 260 X H 100 mm, Part No.048-C, Pioneer Packing, Dixon, KY). Ten windows (27 mm dia.) were cut in the sides of the box (6 and 4 in the long and short sides, respectively) and windows were covered with nylon screen (mesh 500 µm) (Fig. 1A). The bottom of the cage was lined with absorbent paper towel (Fig. 1Aa). The top of the cage was modified with 4 windows (65 mm dia.) covered with nylon screen as above (Fig. 1Ab). Two of the top windows were further modified as water-holding niches by gluing cut outs of plastic containers with screw closing covers over the screen (Fig. 1Ac). Water holding niches were filled with saturated crystals of a water-absorbing polymer (cross-linked potassium polyacrylate / polyacrylamide copolymer, (T-400, The Terawet Co, San Diego, CA) (Fig. 1Be and C). Insects have access to the water crystals through the screen at the bottom of the water niches. A central access cylindrical opening with screw cover was constructed using a cut out of plastic container and fitting into a circular opening with the sides of the container extending to the interior (10 mm) to prevent insects from escaping

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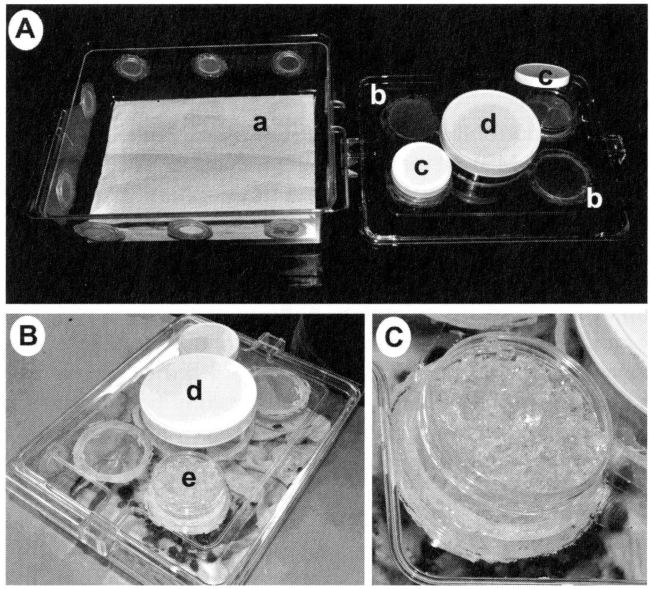

Fig. 1. Cage design. A) Opened cage showing bottom and top sections. The bottom was lined with a paper towel (a) and had a total of 10 lateral windows, 3 per each long side and 2 per short side. Cage tops were modified with aeration windows (b) water niches (c) and with a central access opening (d). B) Closed cage showing the central access closed (d) and an opened water niche filled with saturated water crystals (e). C) Close up of the water niche. The water niches had a screen in the bottom connecting to the inside of the cage allowing access to the water crystals by insect haustellate mouth parts.

(Fig. 1Ad). Three crumpled paper towels were placed inside cages as hiding and/or oviposition substrate in one side of the cage.

Eggs were collected daily by cutting egg masses from the oviposition substrate. Egg masses were placed in large Petri dishes (25 X 150 mm) modified with a screened window (40 mm dia.) in the center of the cover. Dish bottoms were lined with a paper towel.

First instars were transferred to cages as soon as they hatched. Nymphs from first to fourth instar were reared in smaller versions of the cages described in Fig. 1 and were constructed from plastic boxes (L 190 X W 138 X H 95 mm, P. No. 195-C, Pioneer Packing, Dixon, KY) (Fig. 2). Six windows of the 30 mm diameter as in the larger cages were cut in the sides (2 and 1 in the long and short sides, respectively) and covered with nylon screen as above. Two windows were cut out of the top of the cage and covered with nylon screen of the same dimensions as described for the larger cages. One of the windows was modified with the addition of a water niche as above described (Fig. 2). The bottom of the cages was lined with paper towels. Fifth instars were daily transferred to the larger cages used for the adults and provided with 3 crumpled paper towels as hiding substrate.

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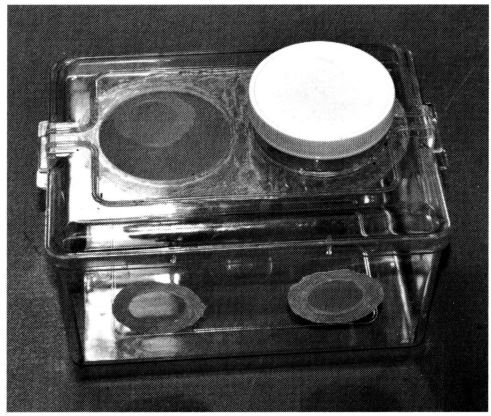

Fig. 2. Smaller version of the rearing cage design. The bottom has only 6 windows, two in each of the larger sides and on in each of the shorter sides. The tom of the cage has only one aeration window and one water niche and no access opening. All windows had the same dimensions as in the larger cage version.

Thirty adults or nymphs per cage were fed a diet consisting of raw peanuts, fresh broccoli and fresh green bean pods. Insects were provided with water from the top of the cage by filling the water-holding niches with saturated crystals of a water-absorbing polymer (cross-linked potassium polyacrylate / polyacrylamide copolymer, (T-400, The Terawet Co, San Diego, CA) (Fig. 1Ab, B-C). Providing water in this manner prevented contamination by insects defecating into the water source by forcing insects to hold themselves to the top of the cage whereas drinking. Gravity induces frass to fall down away from the water source. Vegetables and peanuts were placed directly into the cage and changed every 2 days. Insects were kept in the controlled environment room set at 26°C ± and 67% RH with a 16:8 h (L:D) h photoperiod as described above.

Fifth-instar coloration patterns. Normally, there is a dramatic change in nymphal coloration between the black white-spotted fourth (Fig. 3A & B) to green fifth instars with red abdominal margins and characteristic markings on the abdominal terga (Fig. 3C & D). Occasionally, these coloration changes do not occur, producing an abnormally colored black white-spotted fifth instar (Fig. 3I & J). Intermediate fifth instar coloration patterns (Fig. 3E-H) occurred at a much lower frequency. Coloration patterns of fifth instars were classified into four categories: (1) Green coloration, normal green body with red marks on abdominal ridges and characteristic abdominal markings (three white spots on black background in 2 - 3 abdominal terga separated by red oval spot) (Fig. 3C-D); (2) faded coloration, pale green with white spots on abdominal ridges instead of red, and pale colored characteristic abdominal markings (Fig. 3E-F); (3) intermediate black-green coloration, large areas of thorax or abdomen colored black instead of green and characteristic markings in abdominal terga lacking separating red oval spots (Fig. 3G-H); and, (4) black coloration, displaying fourth instar-like color pattern (Fig. 3I-J).

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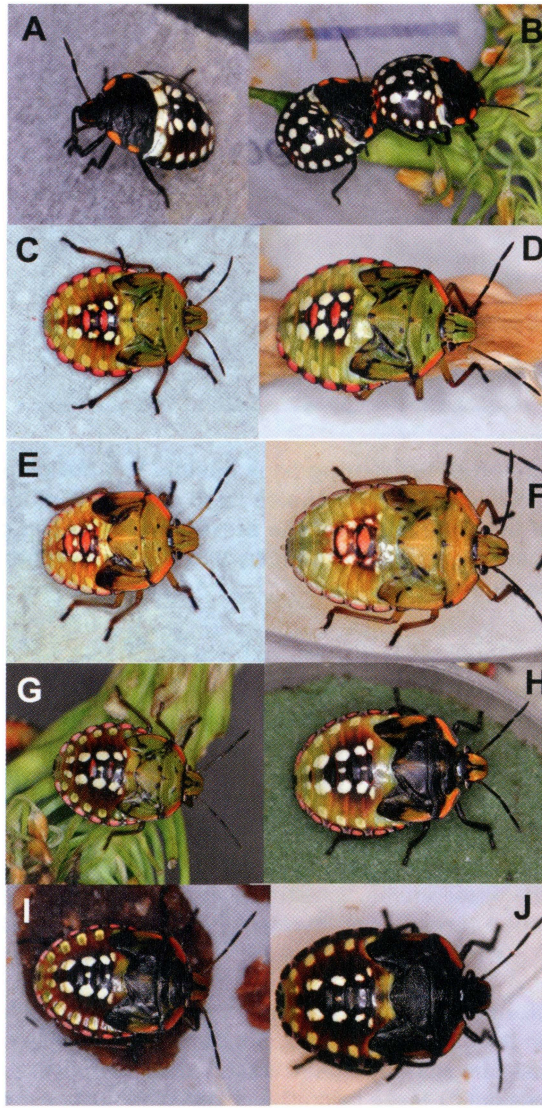

Fig. 3. Coloration patterns observed in the nymphs of *Nezara viridula*. A and B) Normal forth instar coloration pattern. C and D) Normal fifth instar coloration pattern (green). From E to J are coloration patterns of fifth instars considered abnormal. E and F) Faded fifth instar coloration pattern. G and H) intermediate black green fifth instar coloration pattern. I and J) Black fifth instar coloration pattern resembling the normal forth instar coloration pattern.

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Experiment 1 – Influence of nymphal coloration on nymphal survival and adult fecundity. Young nymphs of *N. viridula* from stock colony were allowed to develop in an environmental chamber at 26 ± 1°C, 67 ± 5% RH and 16:8 (L:D) photoperiod. When nymphs reached the fifth instar they were separated according to their coloration pattern as described above (Fig. 3). Three groups of 30, 2-day-old 5th instars of each of the green, black and faded coloration patterns, were placed separately by color in boxes as described in Fig. 1. Food was also provided as described above. Boxes were kept in the environmental chamber at the same conditions described above and allowed to complete development. Dead insects were removed daily from the rearing boxes and daily mortality of each coloration group was recorded. Newly emerged adults developing from nymphs of each coloration group were weighed and recorded. Thirty adults (15 males and 15 females) originating from nymphs of each of these 3 coloration patterns were placed in the adult clean cages. Adult groups were reared at the same conditions and fed as described above. Mortality and oviposition were monitored daily. Egg masses were collected daily and the number of eggs per mass were counted under a stereo-microscope and placed into 18 mm diameter Petri dishes to determine hatching. Data were collected until all adults in the treatments died.

The X^2 test was used to determine whether there was an association between coloration patterns and nymph mortality and adult survival. The Z test was used to compare survival proportions among coloration pattern groups. Egg viability was analyzed using ANOVA. Egg viability proportions were arcsine transformed before analysis. The Tukey-Kramer HSD test was used to compare means among coloration groups. Statistical software used was JMP version 8.0.1 (SAS Institute 2008).

To determine if coloration patterns had an effect on adult weight, additional groups of nymphs of each coloration pattern were reared to complete development at the same conditions described above. Adults resulting from these nymphs were grouped by nymphal coloration pattern and weighed. This process continued until 30 adults from each nymphal coloration pattern were obtained. The weights of adults were recorded and analyzed using ANOVA and means among coloration pattern groups were compared using the Tukey-Kramer HSD test at $\alpha = 0.05$.

Experiment 2 – Transgenerational persistence of coloration patterns. To determine if normal and abnormal coloration patterns persisted across generations, fifth instars were separated by coloration and allowed to reach adulthood. Resulting adults from each coloration pattern group were used to grow independent colonies for each nymphal coloration pattern. The coloration patterns chosen for this experiment were green (Fig. 3C & D), black (Fig. 3I & J), and intermediate black-green (Fig. 3G & H). Individuals of faded coloration (Fig. 3E & F) were not used in this experiment because their numbers diminished until their occurrence became extremely rare in the colonies. Nymphs of each colony were separated by color when they reached the fifth instar. Nymphs were grouped by coloration pattern to obtain adults for the following generation. This procedure was repeated for three generations. Because mortality was extremely high on the black and intermediate colored fifth instars, streptomycin was provided in the water in all the treatments to reduce microbe densities that may be causing some of the mortality (Hirose et al. 2006). Streptomycin sulfate (P. No. S0774, Sigma-Aldrich, Saint Louis, Mo) was dissolved at a concentration of 12.5 mg/100 ml (125 ppm) in reverse osmosis water and presented to the adults in saturated cotton balls in Petri dishes. The normal source of water provided in saturated polyacrylamide (Fig. 1Ab) was maintained free of antibiotic. The antibiotic mixture was

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refreshed twice a week. Egg masses were collected daily, eggs in masses were counted under a stereo microscope and singly placed in rectangular plastic boxes (15 X 10 X 10 cm) to determine hatching and development. Hatchlings were transferred to cages of the smaller version (Fig. 2), fed and watered as described above, and provided with streptomycin sulfate solution in a saturated cotton ball as above (Hirose et al. 2006). Nymphs were allowed to develop to fifth instar at the conditions described above. Progeny from 10 egg masses from each of the 3 adult groups surviving to fifth instars were classified according with their coloration pattern. Green, black and intermediate colored nymphs from the progeny of each adult group were counted and recorded. The proportions of nymphs of each coloration pattern were compared among groups by Chi-square (χ^2) test. Egg viability was compared among groups of adults from different nymphal coloration patterns using ANOVA of arcsine transformations of proportions of viable eggs per egg mass as described above. Means were compared among groups by the Tukey-Kramer HSD test at $\alpha = 0.05$.

Results

Nymphal survival and adult fecundity. *Nezara viridula* from the black coloration group showed significantly higher mortality from fifth-instar to 3-d-old adults than the green coloration group (Table 1). Adults resulting from the black coloration group did not reproduce. Nymphal mortality on the faded coloration group was higher than in the green coloration group, but not significantly different from the black coloration group. Survival from fifth-instar to 3-day-old adults was significantly higher in the green coloration group than in the other 2 color groups ($X^2 = 91.55$, $df = 6$, $P < 0.0001$) (Table 1).

Forty-eight adults in the green coloration group (25 males and 23 females) survived to reproductive age and produced a total of 23 egg masses (Table 1). During the same period, 16 surviving adults (8 males and 8 females) of the faded coloration group produced only 3 egg masses. The 11 surviving adults (5 males and 6 females) in the black coloration group did not produce eggs.

Table 1. Survival of fifth instars displaying different coloration patterns and weight, fecundity, and egg viability of resulting adults.

Coloration pattern	Fifth instar Survival*	Adult weight (mg)**	Egg masses†	Eggs per mass	Egg Viability‡
Green	0.762a	228.7 ± 27.8a	23	80.7 ± 17.8a	0.973a
Black	0.21b	80.7 ± 8.3c	0	-	-
Faded	0.125b	99.4 ± 7.8b	3	47.7 ± 4.0b	0.238b

* To 3-day old adult, initial $n = 63$, 76, and 88 for green, black, and faded color patterns, respectively. Proportions followed by the same letter are not significantly different after Z test at $\alpha = 0.05$.

** N = 30.; Means ± SD followed by the same letter are not significantly different (Tukey-Kramer HSD test; $\alpha = 0.05$).

† Produced per group of 23, 6, and 8 surviving females of the green, black, and faded coloration pattern groups, respectively, for a 15-d period.

‡ Proportions followed by the same letter are not significantly different after Z test at $\alpha = 0.05$.

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Adults from the green coloration group produced a total of 1856 eggs. From those eggs, 1806 first instars hatched, yielding 97.3% egg viability. Adults from the faded coloration group produced only 143 eggs with only 34, or (23.8%) successfully hatching (Table 1).

Adult weight was significantly different among nymphal coloration pattern groups ($F = 265.88$; $df = 2, 44$; $P < 0.0001$) (Table 1).The weight of adults completing development in the green coloration group was significantly higher than in the other two color pattern groups and adult weight of the faded coloration group was significantly higher than in the black coloration group ($F = 265.88$; $df = 2, 44$; $P < 0.0001$) (Table 1).

Trans-generational persistence of coloration patterns. The addition of streptomycin sulfate to the water improved survival of fifth instars with black coloration pattern allowing them to reach adulthood. Groups of adults originating from fifth instars of green, black, and intermediate coloration patterns produced progeny that resulted in fifth instars displaying all 3 coloration patterns (Table 2). Although females originating from green-colored nymphs produced a higher number of green-colored fifth instars, the frequencies of the coloration patterns in the progeny did not differ significantly ($X^2 = 8.11$; $df = 4$; $P = 0.0875$) among adult groups originating from the different nymphal coloration patterns (Table 2). Progeny of adults originating from green fifth instars consisted of 50% green, 24.5% black, and 25.5% intermediate colorations. Adults originating from black-colored nymphs produced 36.5% green, 39.5% black, and 24.3% intermediate colored fifth instars. Adults originating from intermediate colored nymphs produced fewer progeny (a total of 221 eggs compared with 711 and 680 eggs in the green and black groups, respectively) only 7 developing to fifth instars and consisted of 14.3% green, 57.1% black, and 28.6% intermediate colored nymphs (Table 2).

Discussion

Our results show that lack of changes in nymphal coloration between the fourth and fifth instars in *N. viridula* is an indication of health decline. Nymphs that retain forth instar-like coloration after molting to fifth instar as well as those with faded coloration pattern will not produce reproductive adults. Nymphs with faded coloration experienced higher mortality and resulting adults were smaller and their fecundity was dramatically reduced. Fifth-instar coloration patterns could be used as a quick indicator of health and could aid efforts to introduce healthy individuals from the field into the cultures to enhance genetic variability of the colonies. These patterns could also be used as an indicator of colony health and potentially could aid rearing efficiency by eliminating individuals with abnormal coloration during the fifth instar.

Variability in coloration patterns, however, persisted in culture even after selecting fifth instars by coloration pattern. Adults originating from fifth instars with normal coloration pattern produced progeny with abnormal fifth instar coloration in similar proportions as adults originating from nymphs with abnormal coloration. This is an indication that the reasons behind the variation in fifth instar coloration pattern are more complex than disease infection. Other factors yet unknown seem to play a role in coloration pattern changes, but fifth instar coloration is still a good indicator of nymphal health.

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Table 2. Proportion of coloration patterns observed in the progeny of adults originating from green, black, and intermediate nymphal coloration patterns.

	Number of surviving fifth instars per coloration pattern**				Egg		Coloration pattern proportions**,‡		
Adults*	G	B	I	Total	Eggs	Viability†	G	B	I
Green	55	27	28	110	711	0.77 ± 0.29a	0.5	0.245	0.255
Black	27	29	18	74	680	0.66 ± 0.29ab	0.365	0.392	0.243
Intermediate	1	4	2	7	221	0.33 ± 0.36b	0.143	0.571	0.286

* Coloration patterns during fifth stadium.

** Nymphal coloration pattern: green (G), black (B), and intermediate (I).

† Means ± SD followed by the same letter are not significantly different (Tukey-Kramer HSD test of arcsine transformations; $\alpha = 0.05$). Initial $n = 711$, 680, and 221 eggs for the green, black, and intermediate coloration patterns, respectively.

‡ Proportions of nymphal coloration patterns did not differ significantly among progeny of adults originating from green, black, and intermediate colored fifth instars after χ^2 test at $\alpha = 0.05$.

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