Demography of Gut Symbiotic and Aposymbiotic *Nezara viridula* L. (Hemiptera: Pentatomidae)

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ABSTRACT *Nezara viridula* L. is a highly polyphagous and cosmopolitan pentatomid stink bug. Despite its economic importance, aspects of its biology are poorly understood. *N. viridula* has one primary bacterium associated with its gastric caeca, which females provide to offspring by smearing it on the surface of eggs during oviposition. We studied the impact of three temperatures and egg mass surface sterilization on *N. viridula*'s nymphal development rate and reproductive performance. Our results show that maintenance of the symbiont is affected both by temperature and egg mass surface sterilization. We detected the symbiont in 100, 84, and 8.3% of the untreated control insects at 20, 25, and 30°C, respectively, by using polymerase chain reaction. In insects originated from surface sterilized egg masses, the symbiont was never detected at 20 or 30°C and was detected in only 1 of 21 insects at 25°C. Nymphal mean development time decreased with increasing temperature, but there were no differences between the sterilized and control treatments. Sterilized insects at 20°C lived longer than insects in any other treatment but never laid eggs. Life table analysis of *N. viridula* adults showed that net reproductive rate, intrinsic rate of increase, finite rate of increase, and gross reproductive rate were not significantly different among treatments except at 20°C for the surface sterilized treatment. Mean generation time, however, was significantly longer at 20°C (70.96 ± 4.43 d), regardless of the surface sterilization treatment. Our results highlight the effect that temperature has on the maintenance of this symbiosis and its relationship with *N. viridula* host's development and reproduction.

KEY WORDS southern green stink bug, *Buchnera*, Heteroptera, symbiosis

Associations between microorganisms and insects are widespread in nature and can be obligate for the host, for the symbiont, for both, or for neither (Moran 2006). In general, the mutualistic bacteria associated with insects have been placed in two categories: primary (or obligate) and secondary (or facultative) symbionts (Baumann 2005). The best-known relationship is that between the bacterium *Buchnera aphidicola* (Munson) and aphids. *B. aphidicola* is an obligatory symbiont of aphids that cannot live outside its host, colonizing specialized cells called bacteriocytes. It provides nutrients to the aphid host and is vertically (transovarially) transmitted from one generation to another (Munson et al. 1991, Moran et al. 1993). It has been shown that use of antibiotics and heat treatments eliminate *Buchnera*, which in turn affects aphid development and fecundity (Ohtaka and Ishikawa 1991, Douglas 1996, Montllor et al. 2002, Russell and Moran 2006). Facultative symbionts can be horizontally transmitted, creating the potential for multiple acquisitions of the symbionts by different insect species. Additionally, facultative symbionts may confer fitness benefits to their hosts, such as heat stress tolerance (Montllor et al. 2002, Russell and Moran 2006), compensation for loss of *B. aphidicola* (Koga et al. 2003), resistance to parasitoid wasps (Oliver et al. 2003), and resistance to pathogens (Scarbourough et al. 2005). Facultative symbionts can also have negative effects on growth, reproduction, and longevity of the host (Chen et al. 2000).

The southern green stink bug *Nezara viridula* L. (Hemiptera: Pentatomidae) is a highly polyphagous and cosmopolitan insect, occurring on all continents, except Antarctica (Panizzi et al. 2000). Although it is not known definitely, the most likely point of origin of *N. viridula* is the Ethiopian region of eastern Africa (see Todd 1989). In general, stink bugs have been recorded in many crops throughout most of the warmer regions of the world (Todd 1989) and are a major problem in soybean and other crops (Panizzi et al. 2000). Stink bugs attack host plants by inserting their piercing-sucking mouthparts into tissues and introducing digestive enzymes, which cause direct damage to the fruit and may eventually cause premature abscission of fruits. In addition, holes made by their stylets may allow the entrance of microorganisms that

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affect fruit quality (Jones and Caprio 1990, Panizzi et al. 2000).

Buchner (1965) hypothesized that pentatomid stink bugs have an intimate relationship with cecum-associated bacteria. It has previously been shown that *N. viridula* and eight other species of pentatomids harbor a dominant bacterial symbiont in their posterior midgut (ceca or V4 region). The phylogenetic placement of these bacteria indicated they are polyphyletic (Prado and Almeida 2009). However, these nine different symbionts were placed together with *Erwinia* and *Pantoea* species, which are plant-associated bacteria. Female stink bugs vertically transmit the bacteria to their offspring by smearing cells on the egg mass during oviposition, although specific details of this process are unknown (Buchner 1965). Aposymbiotic (symbiont-free) first-instar stink bugs acquire the symbionts when they hatch and probe on the surface of eggs (Buchner 1965, Abe et al. 1995, Prado et al. 2006). First-instar nymphs remain aggregated on the surface of egg masses after hatching, a behavior previously hypothesized to provide protection against desiccation and, potentially, predators (Lockwood and Story 1986). In fact, it has been shown for *N. viridula* that this aggregation behavior results in faster insect development and reduced mortality (Lockwood and Story 1986). However, a similar behavior is also associated with symbiont acquisition: plataspid stink bugs move more as first instars if symbiont-filled capsules (which harbor bacteria essential for survivorship) are removed from their egg masses (Hosokawa et al. 2008). For pentatomids, such as *N. viridula*, a combination of protection from biotic and abiotic stresses in addition to acquisition of symbionts are plausible explanations for this behavior. Prado et al. (2006) showed that growth and mortality of *N. viridula* at a controlled temperature (25 ± 2°C) was not affected by the absence of its gut symbiont. However, deprivation of gut-associated bacteria in other species in the infraorder pentatomomorpha resulted in slowed growth and/or higher nymphal mortality (Abe et al. 1995, Fukatsu and Hosokawa 2002, Hosokawa et al. 2006).

Research on the demography of insects provides important information on factors affecting population growth, but this approach has not been extensively used to study the role of bacterial symbionts in insect host populations. For example, life table construction has been used as a demographic measure to compare efficiency of insecticides on stink bugs (Zanuncio et al. 2005), to compare different rearing techniques and diets for stink bugs (Fortes et al. 2006), and to quantify life history traits at different temperatures (Legaspi 2004). Previous work showed that temperature may interfere with insect reproduction and life history through elimination of the insect's primary symbionts (Ohtaka and Ishikawa 1991). We hypothesized that different temperatures might affect the primary symbiont of *N. viridula* and decrease reproductive performance and subsequent development. In this study, we compare various demographic parameters between symbiotic and aposymbiotic *N. viridula* at three different temperatures.

Materials and Methods

Insect Colonies. *Nezara viridula* adults were collected from multiple locations and host plants on the island of Hawaii and reared in screen cages (28 by 52 by 54 cm) at the USDA–ARS laboratory in Hilo, HI, at 25 ± 2°C and a photoperiod of 14:10 (L:D). *N. viridula* were fed fresh green beans, cabbage, and unsalted roasted peanuts. Adults mated randomly, and wild caught insects were added to the colony every generation. To start the experiments, we collected 200 eggs laid on the same day and randomly assigned them to two treatments: surface sterilized eggs or untreated controls. Under our experimental conditions, *N. viridula* egg masses contained ≈20–30 eggs. In the surface sterilized treatment, egg masses were treated with 10% bleach for 5 min (Prado et al. 2006). The controls were rinsed with water. After treatment, eggs were transferred to 3.8-liter plastic tubs (Rubbermaid, Wooster, OH) with screened lids and supplied with beans, cabbage, and peanuts when they hatched. Containers with eggs were placed in growth chambers set at one of three temperatures: 20, 25, and 30°C. There were four replicates of each temperature-sterilization treatment combination in a factorial design. We replaced food every other day or as necessary. Newly metamorphosed adults were confined in a screen cage (30.4 by 30.4 by 30.4 cm) separate from the nymphs and allowed to mate. Paper towels cut in two inch strips were taped on the walls as an oviposition substrate, and fresh food was added as described above. Once a week, all of the adult cages were checked, removing any egg masses present; adults and nymphs were censused daily to record development and mortality.

Symbiont Detection. We collected fifth-instar (final nymphal stage) and adult *N. viridula* from surface-sterilized and control treatments at all temperatures and stored them at −20°C for symbiont detection. A total of 56 insects (fifth instars and adults) in the control treatment and 26 insects in the surface sterilized treatment were tested. We dissected the insects and collected the V4 section of the midgut. We extracted DNA from insect tissue and used PCR to detect the bacterium as previously described (Prado et al. 2006).

Demography Parameters. We determined the median development time (MDT) by calculating the cumulative proportion of the insect's population that passed through a given stage on a given day (Peterson and Painting 1990). The estimates provided by this method should be relatively robust to influences of mortality. These cumulative proportions were arcsine square root transformed to linearize results with respect to time. We fit separate linear regressions for each stage and used the regression to calculate the number days needed for 50% of the population to pass beyond stage j ($=MDT_j$). We used these methods to calculate MDT from the day that the insects hatched through the final nymphal stage—first instar, second

instar, third instar, fourth instar, and fifth instar—at all temperatures.

We also measured developmental time of nymphal stages as time (d) within each stadium. In addition, we calculated several life table parameters: net reproductive rate (R_0), mean generation time (T), intrinsic rate of increase (r), finite rate of increase (λ), gross reproductive rate (GRR), adult emergence, and oviposition parameters (preoviposition period, oviposition period, total number of eggs) as described by Carey (1993a). For these calculations, we assumed a sex ratio of 50%. Finally, life expectancies were calculated considering longevity (T_x: days lived beyond age x) and survival rate (l_x: survival from age 0 to the beginning of age x) by the approximated method (Southwood 1978): $e_x = T_x/l_x$, described by Carey (1993b).

Statistical Analysis. We used the software R v. 2.6.1 to perform the statistical analyses (R Development Core Team 2007). The total proportion of insects positive for symbiont detection at 20, 25, and 30°C was compared using a logistic regression, with sterilization treatment as a fixed effect and temperature a covariate. For MDT statistical analyses, we used a linear mixed effects model with development stage as a random, repeated measure (Crawley 2005). This method allowed us to compare differences in MDT among temperature and sterilization treatments but not among stages. We log transformed MDT to meet the test assumptions. Significant main effects of treatments were followed up with pairwise t-tests among sterilization treatments and among temperatures. The effect of temperature on life table parameters (R_0, T, r, λ, and GRR), adult emergence (day that the first adult appeared), and oviposition parameters (preoviposition period, oviposition period, total number of eggs) were analyzed using separate one-way analyses of variance (ANOVAs) with five treatment levels (20°C-Control, 25°C-Control, 30°C-Control, 25°C-SS, and 30°C-SS). This approach was used because insect survival was so low in the 20°C SS treatment that the parameters could not be estimated for any of the four replicates. The exception was for adult emergence, for which a 2 by 3 factorial ANOVA was used. We log transformed GRR to meet test assumptions. Significant main effects were followed up by two tests: contrasts between control and surface sterilized and contrasts between 25 and 30°C. Pairwise t-tests were used to compare means among temperatures within each sterilization treatment with Bonferroni adjustments to account for multiple comparisons. For life expectancy statistical analyses, we used a linear mixed effects model with days as a repeated measure (Crawley 2005).

Results

Symbiont Detection. Overall, there was a significant effect of temperature ($z = 2.468$, $P = 0.0136$) and of the interaction between temperature and sterilization treatment ($z = -1.983$, $P = 0.0474$) but not of treatment alone ($z = -0.509$, $P = 0.6104$) on the presence of gut symbionts in *N. viridula.* We detected *N. viridula*'s symbiont in 100% of the insects at 20°C, in 84% of the insects at 25°C, and in 8.3% of the insects at 30°C in the control treatment (Fig. 1). In the surface sterilized treatment, only 1 insect of 11 tested was positive at 25°C. Thus, temperature increments seem to affect symbiont maintenance. Pairwise comparisons between sterilized and nonsterilized treatments showed statistical differences at 20 and 25°C but not at 30°C (Fig. 1).

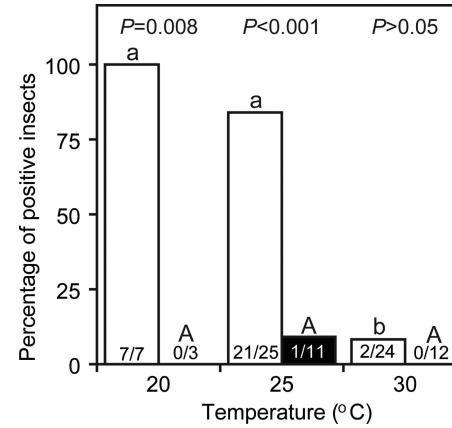

Fig. 1. Percentage of symbiont positive insects at 20, 25, and 30°C. White bars, control; black bars, surface sterilized treatment. Bars with different lowercase letters denote significant difference among temperatures in the control, according to χ^2 follow-up tests. Outcomes of pairwise comparisons between treatments within each temperature are represented by P values above the bars. Numbers at the base of bars: number of symbiont positive/number of tested individuals.

Demography Parameters

MDT. MDT (±SE) for the surface sterilized treatment from the day that the insects hatched until the final nymphal stage (fifth instar) was 62.73 ± 3.59, 39.70 ± 1.09, and 26.54 ± 1.07 d at 20, 25, and 30°C, respectively (Fig. 2). For the control, MDT was

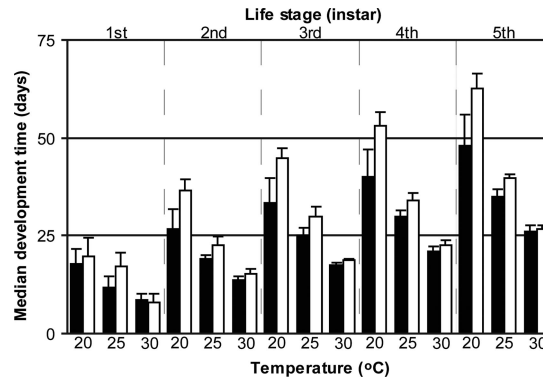

Fig. 2. Mean nymphal development time (±SE) for the five life stages of *N. viridula* at 20, 25, and 30°C. Black bars, control; white bars, surface sterilized treatment.

Table 1. Effect of temperature and surface sterilization on the demographic parameters of *N. viridula*

Factors	20°C		25°C		30°C	
	Control (n^a)	SS (n)	Control (n)	SS (n)	Control (n)	SS (n)
R_0 (♀/♀)[b]	2.13 ± 0.95A (4)	—[c]	2.80 ± 0.83A (4)	2.25 ± 1.58A (2)	1.17 ± 0.53A (4)	1.43 ± 0.85A (2)
T (d)[d]	70.96 ± 4.43A (4)	—	46.59 ± 5.54B (4)	43.00 ± 8.00B (2)	50.67 ± 4.47B (4)	40.68 ± 5.68B (2)
r (♀/♀/d)[e]	0.01 ± 0.01A (4)	—	0.02 ± 0.01A (4)	0.02 ± 0.02A (2)	−0.01 ± 0.01A (4)	0.001 ± 0.017A (2)
l (♀/♀/d)[f]	1.01 ± 0.01A (4)	—	1.02 ± 0.01A (4)	1.02 ± 0.02A (2)	0.99 ± 0.01A (4)	1.001 ± 0.017A (2)
GRR[g]	75.85 ± 50.90A (4)	—	58.35 ± 11.42A (4)	18.24 ± 8.56A (2)	55.85 ± 41.80A (4)	29.34 ± 24.06A (2)

[a] Total no. of replicates.
[b] Net reproductive rate: $R_0 = \Sigma l_x m_x$; means ± SE followed by the same capitalized letters in the same line are not significantly different ($F = 0.5902$, df = 4, $P = 0.677$).
[c] —, no data for the surface sterilized treatment.
[d] Mean generation time: $T = (\Sigma x l_x m_x)/R_0$ ($F = 5.2448$, df = 4, $P = 0.013$).
[e] Instantaneous rate of increase: $r = \ln(R_0)/T$ ($F = 0.5966$, df = 4, $P = 0.673$).
[f] Finite rate of increase: $l = \exp(r)$ ($F = 0.6029$, df = 4, $P = 0.669$).
[g] Gross reproductive rate: $GRR = ?m_x$ ($F = 0.5262$, df = 4, $P = 0.719$).

47.98 ± 7.92, 34.87 ± 2.01, and 26.07 ± 1.56 d at 20, 25, and 30°C, respectively. Results showed a significant effect of temperature ($z = -4.984$, $P = 0.0001$), but we found no significant effect of treatment ($z = -1.4175$, $P = 0.1755$). There was a tendency for control insects to develop faster than surface sterilized treatment at 20 and 25°C but not at 30°C (Fig. 2). The interaction between temperature and treatment was also not statistically different ($z = 1.1193$, $P = 0.2795$). Pairwise comparisons showed significant differences between 20 and 25°C ($t = -2.236$, df = 11, $P = 0.047$) and between 30 and 20°C ($t = -5.413$, df = 12, $P < 0.0001$) but not between 30 and 25°C ($t = -3.216$, df = 11, $P = 0.082$).

Biological Parameters. *Nezara viridula* life table parameters are summarized in Table 1. Comparisons of the effect of temperature on life table parameters between surface sterilized and control treatments showed that temperature did not have an effect on R_0 values; R_0 was highest at 25°C. Mean generation time was significantly different for the temperature treatments. Pairwise comparisons among all three control temperatures showed significantly longer generation time at 20 (70.96 ± 4.43 d) than 25 (46.59 ± 5.54 d, $P = 0.0045$) and 30°C (50.67 ± 4.47 d, $P = 0.0131$). Comparisons between the surface sterilized treatment and control treatment and between 25°C and 30°C did not show significant differences. The life table parameters r, λ, and *GRR* were not significantly different in any of the treatments. Time of adult emergence was significantly different between the temperature treatments; pairwise comparisons showed a significantly longer time to adult development ($t = 3.278$, $P = 0.0074$) at 20°C, but did not show any significant difference between surface sterilized (51.67 ± 4.10 d) and control (38.25 ± 6.61 d) treatments (Table 2). The parameters preoviposition period, oviposition period, and number of eggs laid were not significantly affected by temperature or sterilization treatment (Table 2). In the 20°C of the surface sterilized treatment we could not calculate the life table parameters because of the high mortality of the nymphs. In addition, adults emerged in only two replicates, and never laid eggs for the surface sterilized at 20°C treatment. Females laid more eggs at 25°C in the control (243.50 ± 63.78) and surface sterilized (174 ± 107) treatments than at 20 and 30°C, but the means were not significantly different.

The effect of temperature on *N. viridula*'s life expectancy is presented in Fig. 3. The average life expectancy showed no differences between the survivorship and longevity among temperatures ($t = -0.8603$, df = 17, $P = 0.4016$), treatments ($t = 0.0334$,

Table 2. Effect of temperatue and surface sterilization on adult emergence and reproductive performance of *N. viridula* females

Factors	20°C		25°C		30°C	
	Control (n^a)	SS (n)	Control (n)	SS (n)	Control (n)	SS (n)
Adult emergence[b]	38.25 ± 6.61A (4)	51 ± 3.5A (2)	27.75 ± 1.25B (4)	30.00B (2)	20.50 ± 1.38B (4)	20.67 ± 2.73B (3)
Preoviposition period[c]	28.25 ± 6.80A (4)	—	14.75 ± 3.66A (4)	13.00 ± 8.00A (2)	29.00 ± 3.39A (4)	17.00 ± 1.00A (2)
Oviposition period[d]	13.75 ± 6.75A (4)	—	12.50 ± 4.84A (4)	7.00A (2)	10.25 ± 3.25A (4)	12.00 ± 5.00A (2)
Number of eggs[e]	170.50 ± 65.78A (4)	—	243.50 ± 63.78A (4)	174.00 ± 107.00A (2)	118.00 ± 45.47A (4)	107.00 ± 49.00A (2)

[a] Total no. of replicates.
[b] Significant difference among treatment combinations ($F = 6.6604$, df = 5, $P = 0.003$); means ± SE followed by the same capitalized letters in the same line are not significantly different.
[c] No significant difference among treatment combinations ($F = 2.1628$, df = 4, $P = 0.141$).
[d] No significant difference among treatment combinations ($F = 0.1957$, df = 4, $P = 0.935$).
[e] Total no. of eggs laid during the entire female lifetime. No significant difference among treatment combinations ($F = 0.7238$, df = 4, $P = 0.594$).

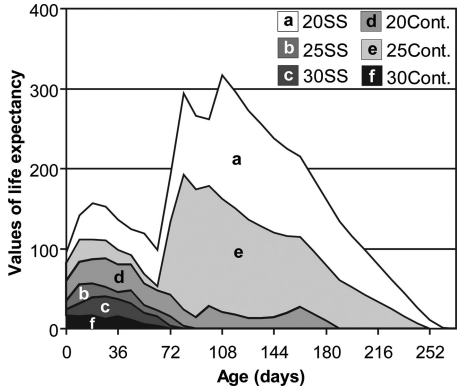

Fig. 3. Mean values of life expectancy for surface sterilized (SS; a–c) and control (Cont.; d–f) treatments at 20, 25, and 30°C.

df = 17, $P = 0.9737$), and the interaction ($t = -0.02$, df = 17, $P = 0.9839$). Trends showed that the average life expectancy of insects at 30°C in both the surface sterilized and control treatments and insects of the surface sterilized treatment at 25°C were low and similar. At 20°C, adults of the surface sterilized treatment tended to have the highest life expectancy and the longest longevity around 261 d, but they never laid eggs.

Discussion

The effects of temperature and egg mass surface sterilization on *N. viridula*'s development and maintenance of a gut symbiont were studied. In general, our results showed that the symbiont's maintenance in *N. viridula* was directly affected by surface sterilization of the egg masses and high temperature (30°C). Heat can negatively impact the population of the aphid primary symbiont *B. aphidicola*, causing host infertility (Montllor et al. 2002). Subjecting immature aphids to high temperatures causes them to lose their obligatory symbiont (Montllor et al. 2002, Russell and Moran 2006), but in some cases, this can be compensated for by the presence of facultative symbionts (Koga et al. 2003). In the case of *N. viridula*, it is unknown if any mechanism exists to compensate for the lack of its symbiont at high temperatures. Further studies are necessary to clarify the relationships observed here and to determine the impact of fluctuating temperatures on this association under field conditions. Panizzi (2002) reported that *N. viridula* is expanding in tropical regions toward warmers areas. If this trend continues, it may have an important implication for *N. viridula* life history, because an upper temperature threshold may exist beyond which the maintenance of its gut symbiont is likely, potentially reducing insect fitness. These considerations need to be studied given current climate change forecasts (Walther et al. 2002).

Nezara viridula's nymphal developmental time was shortened with increasing temperature, similarly to previous reports (Harris and Todd 1981, Vivan and Panizzi 2005). Our experiments were not able to decouple the relative effect of high temperature on the insect host alone and loss of the gut symbiont. Surface sterilization did not significantly affect nymphal development time, but there was a tendency toward slower nymphal development time in insects without symbionts (surface sterilized treatment). *N. viridula* that originated from surface sterilized egg masses in the 20°C treatment had the longest nymphal developmental time and the longest mean generation time. In addition, the insects in the surface sterilized treatment at 20°C had the highest values of life expectancy, the longest time to adult emergence, and the greatest longevity, yet adults ($n = 13$) never laid eggs. These results suggest that the symbiont may have an important role in determining development time, survivorship, longevity, and generation time of *N. viridula.* Thus, despite the fact this bacterium is vertically transmitted, further work will be necessary to identify its role in *N. viridula*'s biology. Our data, however, indicate that symbiont acquisition occurs during the first instar and that the aggregation behavior pentatomid nymphs exhibit soon after hatching is at least partly driven by the need to acquire these bacteria from the surface of eggs.

Several authors studied nymphal duration of *N. viridula* (Jones and Brewer 1987, Panizzi and Saraiva 1993, Noda and Kamano 2002), but in all of them, they ignored the duration of the first instar, only counting the days that the insects took to develop from second instar to adults. Here, we found that entire nymphal developmental time for *N. viridula* was comparable to previous studies (Ali and Eweiss 1977, Harris and Todd 1980). Similar results for the biological parameters observed among temperatures in this study may be caused by a fitness cost incurred by the insect because of the loss of the symbiont at 30°C. In addition, at lower temperatures, the symbiont's absence had a direct effect on the host by inhibiting reproduction. Additionally, *N. viridula*'s diet used in this research should be considered, although it was consistent across all treatments. We used green beans, which are a good source of carbohydrates and protein, and it has been shown that, together with peanuts, they are adequate for *N. viridula*'s development (Todd 1989). Such an unusually rich diet may have masked differences in fitness among the treatments tested here.

This research highlights the importance of the use of demographic studies to show the effect of bacterial symbionts in the development of insects. The results suggest that the absence or presence of the symbiont and/or its interaction with abiotic factors must be taken into account to understand the ecology of *N. viridula* populations. Additionally, *Nezara viridula* is a widespread important pest, and an understanding of factors associated with its population's performance is important for predicting economic impacts. Finally, research with other pentatomid species is needed to

determine the importance of these gut symbioses for the biology of their stink bug hosts.

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