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Role of symbiotic gut bacteria in the development of *Acrosternum hilare* and *Murgantia histrionica*

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Abstract

The green stink bug, *Acrosternum hilare* (Say), and the harlequin bug, *Murgantia histrionica* (Hahn) (both Heteroptera: Pentatomidae), are pests of many economically important crops. Although both species have been studied extensively, until recently their relationships with symbiotic gut bacteria have remained unknown. The endosymbionts may be important, as other pentatomomorphan species harbor vertically transmitted gut bacteria that play an important role in the biology of their host insects. We report the role of gut symbiotic bacteria on the development and fitness of *A. hilare* and *M. histrionica* by comparing control insects with individuals that hatched from surface sterilized egg masses. We studied the life history of *A. hilare* and *M. histrionica* under laboratory conditions at 23 ± 2 °C and L16:D8, measuring nymphal mortality and development time, and estimating other demographic parameters. We also evaluated egg masses, nymphs, and adults of both species with diagnostic PCR primers for the presence of specific gut symbiotic bacteria for both treatments. Our results show that egg mass surface sterilization eliminates or reduces the prevalence of this bacterium-insect symbiosis in both species. *Acrosternum hilare*'s development time, survivorship, and other demographic parameters were negatively affected by surface sterilization of egg masses. Conversely, *M. histrionica*'s survivorship was marginally increased by clearing its symbiont infection during the first generation; however, mean generation time was significantly longer. Our data indicate that the degree of mutualism in this association for pentatomid species is variable, given that *A. hilare* requires its symbiont for adequate development and survival, whereas *M. histrionica*'s symbiont may not be required for development and survival under the experimental conditions used.

Introduction

Microbial symbionts have an essential role in the biology and evolution of many insect groups (Baumann, 2005). Insects are often hosts to microbial symbionts and have remarkably diverse associations with microorganisms. These relationships have been of interest to biologists partly because of their diversity, ranging from obligate beneficial mutualisms to harmful parasitic associations (Buchner, 1965). Symbiotic associations among insects of the order Hemiptera and their associated bacteria are present in three of its suborders: Sternorrhyncha (e.g., aphids, mealybugs, whiteflies, and psyllids), Auchenorryhyncha (e.g., spittlebugs, planthopers, leafhoppers, and treehoppers), and Heteroptera (true bugs) (Moran et al., 1993; Fukatsu & Hosokawa, 2002; Baumann, 2005; Hosokawa et al., 2006). The suborders Sternorrhyncha and Auchenorryhyncha include insects of economic importance that cause direct plant damage or transmit plant pathogens. Heteroptera also include economically important insects that feed on plants and blood as well as on other insects, with a few taxa being vectors of pathogens (Panizzi et al., 2000).

The best explored symbiotic relationship is that of the bacterium *Buchnera aphidicola* Munson et al. and the aphid *Acyrthosiphon pisum* (Harris) (Moran et al.,

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1993). In addition, mealybugs, whiteflies, and psyllids also harbor their own obligatory endosymbionts that are vertically (maternally) transmitted to offspring (Baumann, 2005). In Heteroptera, some symbionts inhabit the gut lumen (Reduviidae) or the gastric caeca (e.g., Pentatomidae, Plataspidae, and Alydidae) and are not transovarially transmitted (Buchner, 1965; Durvasula et al., 1999; Fukatsu & Hosokawa, 2002; Prado et al., 2006). These bacterial symbionts are orally transmitted through infection of materials associated with the egg masses or fecal pellets (Buchner, 1965). The Japanese stink bug, *Megacopta punctatissima* (Montandon) harbors bacterial symbionts in their gastric caeca (Fukatsu & Hosokawa, 2002). *Megacopta punctatissima* females attach small capsules filled with symbionts to their egg masses. After hatching, the nymphs immediately probe these capsules and acquire the symbionts. It has been shown that plataspids and their symbiont have cospeciated and that the bacterium's removal causes retarded growth, mortality, and sterility of the insects (Fukatsu & Hosokawa, 2002; Hosokawa et al., 2006). Conversely, other work with *Riptortus clavatus* (Thunberg) and *Leptocorisa chinensis* (Dallas) demonstrated that their dominant symbionts are not vertically transmitted by females, but are environmentally acquired each generation by 1st instars from soils and are not monophyletic (Kikuchi et al., 2005, 2007). Insects of the family Pentatomidae (Pentatomomorpha) harbor a dominant bacterium in the last section of the midgut, where the gastric caecum is located (Prado & Almeida, 2009). Symbionts are smeared on the top of the egg masses by females while ovipositing and newly hatched nymphs probe on the egg surface to acquire them (Buchner, 1965). This unique model of vertical transmission, which includes the lack of transovarial transmission, adds an extra challenge to the symbiont: to survive outside its host.

Despite their potential importance, symbiotic associations in pentatomid insects have not been subjected to detailed study. Abe et al. (1995) showed that individuals from surface sterilized egg masses of *Plautia stali* Scott did not reach the adult stage. Development and biology of *Nezara viridula* (L.), however, was not affected by the removal of their symbionts under laboratory conditions (Prado et al., 2006). In addition, high temperatures have been shown to negatively impact retention of symbionts by *N. viridula* (Prado et al., 2009). Phylogenetic analyses of the symbionts of nine species of stink bugs showed that they are polyphyletic. Pentatomid symbionts were phylogenetically grouped with the plant pathogens *Erwinia* and *Pantoea* (Prado & Almeida, 2009). One hypothesis for these data is that the symbionts may be replaced with taxonomically similar bacteria over evolutionary time and that different species of insects may have a different level of dependency on this relationship. Accordingly, one would expect that gut symbionts are required for adequate development of certain pentatomid species, whereas no obvious benefits of the association will be observed for other taxa. In this study we investigated the role that vertically transmitted symbionts have on the development and reproduction of two pentatomids, the green stink bug, *Acrosternum hilare* (Say), and of the harlequin bug, *Murgantia histrionica* (Hahn).

Materials and methods

Rearing of insects

Adults of *A. hilare* used to start our colony were kindly provided by Kent Daane (University of California, Berkeley, CA, USA) and were transferred to our laboratory during the summer of 2006. *Murgantia histrionica*'s egg masses were kindly shipped from Jocelyn Millar's laboratory at the University of California at Riverside to Berkeley. We reared adults of both species in wood frame cages inside an insectary room at 23 ± 2 °C and L16:D8. Three sides of the cages were made of cloth-mesh material. Females used the cloth-mesh wall and strips of paper towel that we hung on strings inside the cage as oviposition surfaces. The colonies were cleaned and the food was replaced twice a week or when necessary. *Acrosternum hilare*'s diet consisted of green beans, broccoli heads, and roasted peanuts, and *M. histrionica*'s diet consisted of broccoli and cauliflower heads. Green beans, broccoli, and cauliflower were purchased at an organic grocery store at Berkeley, and washed and dried before used. Egg masses from lab colonies were used to initiate the experiments. For all the experiments, insects were placed in plastic containers lined with filter paper and fed only on green beans (*A. hilare*) or broccoli (*M. histrionica*). Adults were left in the plastic containers and allowed to lay eggs. Egg masses were collected every day and placed in plastic containers to start the next generation.

Diagnostic PCR

We extracted DNA from whole insects and egg masses using a commercial kit (Qiagen, Valencia, CA, USA) as described previously (Prado et al., 2006). Preliminary tests showed no difference in the rate of symbiont detection by macerating whole insects or only the gastric caeca (also known as V4 region of the midgut). Egg masses were sampled the day after 1st instars left the surface of eggs and moved to the green beans or

broccoli. Specific primers were designed based on the symbiont's partial 16S rRNA gene sequence (Prado & Almeida, 2009). We designed the forward primer SP-Ah1 (5′-GAGGCTTAATACGCTTCG-3′) and the reverse primer SP-Ah2 (5′-CAAGGAAACAACCTC-CAG-3′) for *A. hilare*, and the forward primer SP-Mh1 (5′-CGCATAATGTTTCACAACC-3′) and the reverse primer SP-Mh2 (5′-CACATCTCAAGGATACAAC-3′) for *M. histrionica*. We used the same PCR cycle as described previously (Prado et al., 2006), except that the annealing temperature for both of these primer sets was 58 °C.

Effect of egg mass structure on symbiont detection, mortality, nymphal development, and reproduction in *Acrosternum hilare*

We optimized the protocol previously used for *N. viridula* (Prado et al., 2006) by increasing the 5 min soak in 10% bleach (6% sodium hypochloride) for egg masses to 7 min. Additionally, we set up a preliminary experiment to test the effect that our surface sterilization treatment had on egg mass structure of *A. hilare*, once the eggs would separate from the egg masses after soaking them in the bleach solution. This was done because some authors proposed that separation of the eggs during sterilization would increase mortality and affect the insects' development (Lockwood & Story, 1985). We tested three treatments (5 egg masses/treatment): surface sterilization, physical separation of eggs, and intact egg masses. The surface sterilization treatment consisted of dipping the egg masses for 5 min in alcohol (100%) followed by 7 min in 10% bleach, approximately 2 days before nymphal hatch. The next treatment consisted of physically separating the eggs 2 days before the insects hatched. We used the intact egg masses as a control treatment. We monitored the insects every 5 days after hatch and calculated nymphal mortality, median development time (± SE), and the following demographic parameters: net reproductive rate ($R_0 = \sum l_x m_x$), mean generation time [$T = (\sum \times l_x m_x)/R_0$], intrinsic rate of increase ($r = \ln(R_0)/T$), finite rate of increase ($\lambda = e^r$), doubling time ($DT = \ln 2/r$), and gross reproductive rate ($GRR = \sum m_x$), as described in Carey (1993). To meet the assumptions of normality and homogeneous variance we log transformed the data of the GRR parameter before the analysis. In addition, we recorded adult emergence (day that the first adult appeared) and several oviposition parameters (pre-oviposition period, oviposition period, and number of egg masses) (Carey, 1993). Because counting was done on 5-day intervals, some variability was expected in the estimation of these parameters. We evaluated symbiont retention by testing the egg masses, 2 fifth instars, and an adult male and female in each replicate of all treatments using PCR specific primers.

Effect of egg mass sterilization on symbiont retention and insect fitness

We surface sterilized 20 egg masses of *A. hilare* as described above. We used 20 egg masses as a control. For *M. histrionica*, we surface sterilized 15 egg masses as described above and used 15 egg masses as a control in the first generation. For the second generation, we collected 14 egg masses that originated from the control treatment and 9 egg masses that originated from the surface sterilization treatment and were not surface sterilized.

Symbiont retention. The egg mass, 2 fifth instars, and an adult female and male of *A. hilare* of each replicate were used for detection of the symbiont in samples in each treatment. A total of 20 egg masses, 40 fifth instars, and 40 adults of *A. hilare* were tested, except in the surface sterilization treatment where mortality was high. For *M. histrionica*, the egg mass, 2 third instars, and an adult male and female also of each replicate were used for DNA extraction and detection of the symbiont (as above) in each of the two generations in each treatment. We sampled approximately 15 egg masses, 30 third instars, and 30 adults of *M. histrionica* in each treatment during the first generation. At the second generation, 14 egg masses, 28 third instars, and 28 adults of *M. histrionica* were tested as a control. In the surface sterilization treatment we tested 9 egg masses, 18 third instars, and 18 adults of *M. histrionica*. We compared the proportion of symbiont-positive insects between sterilization treatments and among the three stages using a 2 × 3 contingency table. To test for an effect on symbiont maintenance we used analysis of deviance (i.e., generalized linear model with binomial error) with insect stage and sterilization treatment as fixed effects (Crawley, 2005). Follow-up χ^2 tests were used to assess the significance of the differences between control and surface sterilization treatment within each of the three life stages: egg masses, nymphs, and adults (Crawley, 2005).

Insect fitness. We considered each egg mass as a replicate. We counted the insects in each replicate every 5 days. We measured nymphal developmental time and calculated the median development time (± SE) as described by Peterson & Painting (1990). We executed all the statistical analyses using the software R v. 2.6.1 (R Development Core Team, 2006). For statistical analysis of the median development time, we used a linear mixed effects model with sterilization treatment as a fixed effect

and development stage as a random, repeated variable (Crawley, 2005). We used a Cox proportional hazards model with censoring of the individuals that were removed for PCR to conduct a survival analysis. For this, we compared Kaplan–Meier survival curves for control and surface sterilization treatments in both *A. hilare* and *M. histrionica*. Finally, we calculated several life-history parameters for these laboratory populations, including R_0, T, r, λ, DT, GRR, adult emergence, pre-oviposition period, oviposition period, and number of egg masses (median ± SE) as described earlier (Carey, 1993). We calculated confidence intervals rather than ANOVA to compare *A. hilare*'s biological parameters between control and surface sterilization treatments because in the surface sterilization treatment we had only one repetition for the comparisons. For *M. histrionica*, all demographic and oviposition parameters were analyzed using separate one-away ANOVA with three treatment levels (control-generation 1, control-generation 2, and surface sterilized-generation 1). To meet the assumptions of normality and homogeneous variance we log transformed the data of the GRR parameter for the analysis. We used one-way ANOVA to compare the total numbers of eggs laid per female in three treatments (*A. hilare*-control, *M. histrionica*-control, and *M. histrionica*-surface sterilized) and to compare the total numbers of eggs/female of *M. histrionica* in control and surface sterilization treatments during two generations. Lastly, follow-up pairwise t-tests were used to compare the mean numbers of eggs laid among *A. hilare*-control, *M. histrionica*-control, and *M. histrionica*-surface sterilized.

Results

Effect of egg mass structure on symbiont detection, mortality, nymphal development, and reproduction in *Acrosternum hilare*

The longer bleach treatment caused the eggs in the masses to separate from the egg mass. Thus, we analyzed the significant effect of separating individual eggs when compared with surface sterilization of eggs on *A. hilare*'s development, hypothesizing that egg mass structure does not impact stink bug fitness. Insects that originated from the surface sterilization treatment took longer to develop from first to fifth instar (110 days) ($t = -2.181$, d.f. = 41, $P = 0.035$) (Figure 1). However, there was no developmental time difference between the insects which originated from the physically separated eggs (39.8 ± 10.3 days) and the control (51.4 ± 0.5 days). The total proportion of symbiont-positive individuals for *A. hilare* was lower ($\chi^2 = 40.70$, d.f. = 2, $P<0.0001$) in the surface sterilization treatment (3 positives out of 22 samples) than in the physically separated eggs (22 positives out of 23 samples) and the control (23 positives out of 27 samples), but we found no difference in the detection of the symbiont between physically separated eggs and control eggs. We did not detect any significant effect on nymphal cumulative mortality of *A. hilare* ($F_{2,12} = 1.65$, $P = 0.233$) between control (76.7 ± 7.5%), physically separated (56.0 ± 10.2%), and surface sterilization (74.3 ± 8.5%) treatments. In addition, R_0, T, r, λ, GRR, adult emergence, pre-oviposition period, oviposition period, and number of egg masses could only be statistically analyzed between physically separated and surface sterilized eggs due to the lack of repetition of the control treatment (Table 1). These results support our hypothesis that, under our experimental conditions, egg mass structure has no impact in *A. hilare*'s survival and reproduction.

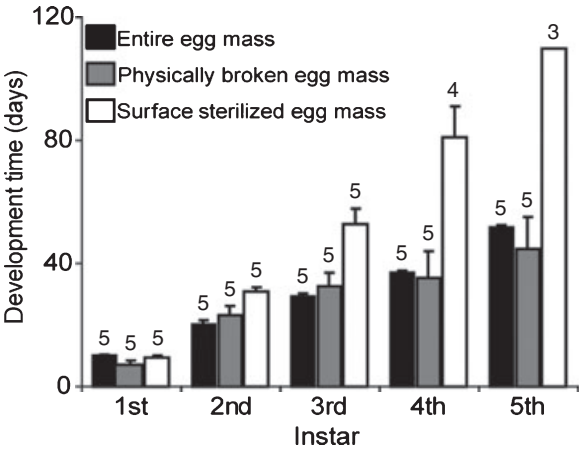

Figure 1 Cumulative median nymphal development time (+ SE) of *Acrosternum hilare* comparing the role of egg mass integrity on insect development. Numbers above bars represent the total number of egg masses tested.

Effect of egg mass sterilization on symbiont retention and insect fitness

Symbiont retention. We detected *A. hilare*'s symbiont in most individuals in the control treatment (Figure 2A). In the surface sterilization treatment, however, we detected the gut symbiont only in 30, 9.1, and 0%, of egg masses, nymphs and adults, respectively. These rates were significantly lower than the control for nymphs ($\chi^2 = 13.61$, d.f. = 1, $P<0.001$) and adults ($\chi^2 = 15.77$, d.f. = 1, $P<0.0001$). No observations were made for the second generation (see below).

Overall, there was a significant effect of generation ($\chi^2 = 6.37$, d.f. = 1, $P = 0.012$) and treatment ($\chi^2 = 76.01$, d.f. = 1, $P<0.0001$) on symbiont maintenance in *M. histrionica* (Figure 2B). Only in the second

Table 1 Effect of physical separation of the eggs and surface sterilization of egg mass on demographic and oviposition parameters of *Acrosternum hilare*

Parameters	Entire egg mass (nc/nf[1] = 1/7)	Physically separated egg mass (4/10)	Surface sterilized egg mass (2/3)	$F_{1,4}$	P
R_0 (♀/♀)	8.47[2]	25.97 ± 5.62	0.98 ± 0.02	8.80	0.04
T (days)	96.66	111.45 ± 2.48	172.94 ± 27.94	12.33	0.024
r (♀/♀/day)	0.02	0.03 ± 0.002	0 ± 0.0001	77.06	<0.001
λ (♀/♀/day)	1.02	1.03 ± 0.002	1 ± 0.0001	75.21	<0.001
GRR	85.36	89 ± 15.77	9 ± 4.25	11.23	0.029
Adult emergence (days)	50	50 ± 0.85	89 ± 13.50	20.91	0.01
Pre-oviposition period (days)	35	34 ± 3.15	78 ± 7.50	44.14	0.003
Oviposition period (days)	45	71 ± 6.25	15 ± 10.00	25.23	0.007
No. of eggs/egg mass	27	25 ± 3.76	8 ± 1.63	8.22	0.046

F refers to the statistical comparison between the mean parameter values of physically separated vs. surface sterilized egg masses (one-away ANOVA).
[1]Number of cohorts used for the calculation of the parameters/total number of females by the time of the first oviposition in the replicates.
[2]Data censused every 5 days.

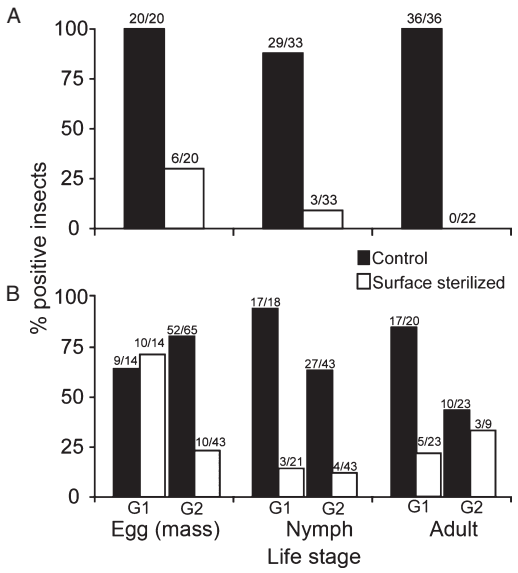

Figure 2 Percentage of symbiont-positive individuals for (A) *Acrosternum hilare* and (B) *Murgantia histrionica*. G1, first generation; G2, second generation. Numbers above columns represent the proportion of positive samples by PCR/total tested.

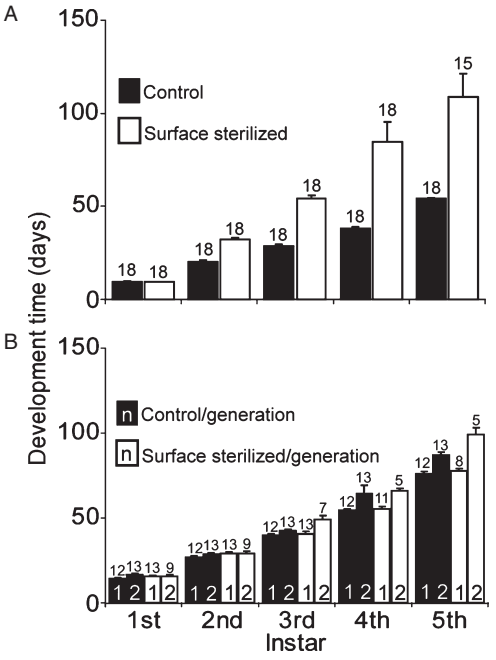

Figure 3 Cumulative median instar development time (+ SE) from egg to completion of each instar of (A) *Acrosternum hilare* and (B) *Murgantia histrionica*. Numbers in the columns indicate generation. Numbers above columns represent the total number of samples tested.

generation infection rate of *M. histrionica*'s eggs was significantly higher in the control treatment than in the surface sterilized egg masses ($\chi^2 = 9.22$, d.f. = 1, P = 0.0024). In nymphs, infection rate in the control was significantly higher than in the surface sterilization treatment in both the 1st ($\chi^2 = 6.74$, d.f. = 1, P = 0.0095) and 2nd ($\chi^2 = 11.55$, d.f. = 1, P = 0.0007) generation. In addition, adults of the control treatment had a significantly higher infection rate than the surface sterilization treatment ($\chi^2 = 4.43$, d.f. = 1, P = 0.035) during the first but not the second generation.

Table 2 Effect of egg mass surface sterilization on demographic parameters of *Acrosternum hilare* (median ± 95% confidence interval; n = 11) and *Murgantia histrionica* (median ± SE). G1, generation 1; G2, generation 2

Species	Treatment/ generation	nc/nf[1]	R_0 (♀/♀)	T (days)	r (♀/♀/day)	λ (♀/♀/day)	DT (days)	GRR
A. hilare	Control/G1	11/38	21.59 ± 7.71	135.99 ± 19.56	0.02 ± 0.004	1.02 ± 0.004	34.48 ± 7.87	81 ± 20.79
	Surface sterilized/G1	1/1	0.40	197	−0.005	0.995	–	4
M. histrionica[3]	Control/G1	8/16	12.17 ± 2.94	128.49 ± 14.52a	0.02 ± 0.003	1.02 ± 0.003	54.33 ± 15.32	31 ± 6.04
	Control/G2	4/5	6.19 ± 1.99	147.71 ± 7.21a	0.01 ± 0.003	1.01 ± 0.003	114.70 ± 63.11	17 ± 4.46
	Surface sterilized/G1	6/14	9.39 ± 1.60	185.16 ± 19.09b	0.01 ± 0.001	1.01 ± 0.001	60.31 ± 3.65	25 ± 3.74
	Surface sterilized/G2	0/0	–[2]	–	–	–	–	–
	$F_{2,15}$		1.21	3.56	2.28	2.28	1.29	1.02
	P		0.326	0.054	0.136	0.136	0.305	0.382

[1]Number of cohorts used for the calculation of the parameters/total number of females by the time of the first oviposition in the replicates.
[2]No data were collected.
[3]Means for *M. histrionica* followed by different letters within a column are significantly different (one-away ANOVA and pairwise t-tests for post-hoc comparisons).

Insect fitness. Acrosternum hilare. Nymphal development time was significantly longer in the egg mass surface sterilization treatment (108.8 ± 12.7 days) than in the control treatment (54.1 ± 0.6 days) (t = −4.02, d.f. = 158, P<0.0001) (Figure 3A). Reproduction was negatively affected by surface sterilization of the egg masses (Table 2). From the initial 20 egg masses in the surface sterilization experiment only three females reached the adult stage, and one female laid only 1 egg mass (Figure 4). In the control treatment, from the initial 20 egg masses a total of 38 females laid 147 egg masses. Overall mortality during the nymphal period averaged 48 and 14% in the surface sterilization and control treatments, respectively, with the highest mortality in both treatments occurring in the second instars.

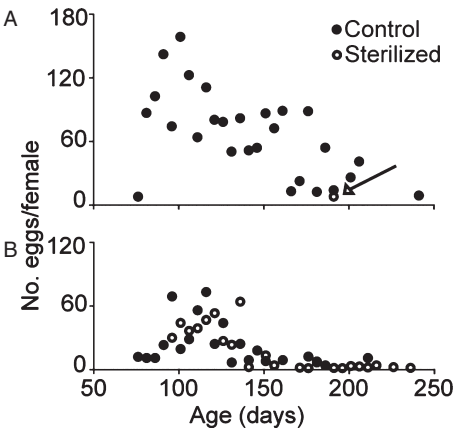

Figure 4 Total number of eggs laid per (A) *Acrosternum hilare* and (B) *Murgantia histrionica* female during the first generation in the control and surface sterilization treatments. The arrow indicates the only egg mass laid in the surface sterilization treatment of *A. hilare*.

Murgantia histrionica. Median nymphal development time (± SE) from eggs to fifth instar in the surface sterilization treatment (SS) was not significantly different from the control in the first (SS vs. control: 77.6 ± 1.6 vs. 76.0 ± 1.1 days) or second (99.0 ± 4.4 vs. 87.1 ± 1.7 days) generation (t = 0.45, d.f. = 202, P = 0.65) (Figure 3B). The 12 egg masses followed in the control treatment yielded 16 females that laid 69 egg masses in the first generation, and five females laid 16 egg masses in the second generation. In the surface sterilization treatment, 14 egg masses yielded 14 females that laid 53 egg masses in the first generation; all six females that developed in the second generation were used for detection of the symbiont. Survivorship was slightly increased by the absence of its symbiont (χ^2 = 5.8, d.f. = 1, P<0.016) (Figure 5). Comparisons between treatments and generations demonstrated significant differences in survivorship between generations (z = 2.31, P<0.021), but not treatment (z = −1.42, P = 0.15). Mean generation time (T) was longer in the surface sterilization treatment (t = 2.66, d.f. = 15, P = 0.0018) than in the control treatment (Table 2). In general, the second generation in the control treatment showed a tendency of longer T and DT, with smaller

values of R_0, r, λ, and GRR than in the first generation. As shown in Table 3, pre-oviposition time was longer for the second generation of the control treatment than the first generation and surface sterilization treatment ($F_{2,15} = 7.98$, $P = 0.0044$).

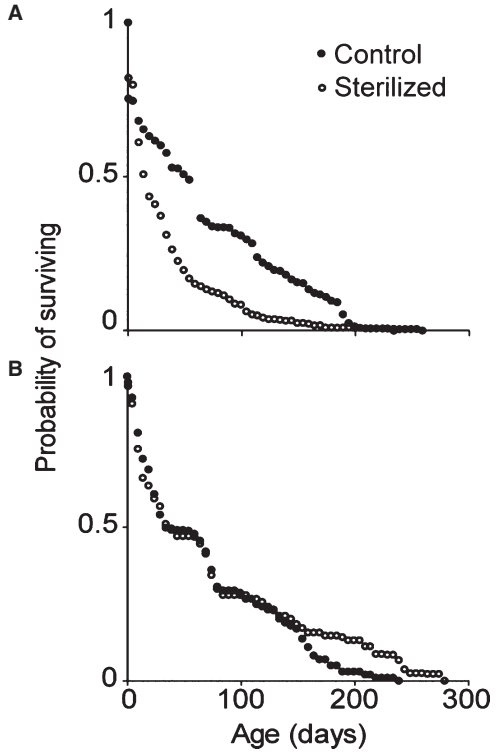

Figure 5 Survivorship curves for (A) *Acrosternum hilare* and (B) *Murgantia histrionica*.

Acrosternum hilare vs. Murgantia histrionica. Survival analysis showed a significant effect of surface sterilization treatment ($z = 6.291$, $P<0.0001$), and a significant interaction between the sterilization treatment and species ($z = -5.440$, $P<0.0001$). Survival of *A. hilare* was negatively affected by the absence of its symbiont ($\chi^2 = 46$, d.f. = 1, $P<0.0001$), whereas the absence of its dominant symbiont marginally increased survival of *M. histrionica* (Figure 5). We also plotted the total number of eggs/female laid during adulthood (Figure 4). Comparing three treatments, *A. hilare*-control, *M. histrionica*-control, and *M. histrionica*-surface sterilized, we showed that *A. hilare* laid more eggs in the control, treatment (139 ± 108 eggs) than *M. histrionica* in the control (37 ± 30) or surface sterilization (23 ± 17) treatment ($F_{2,68} = 20.08$, $P<0.0001$).

Discussion

We showed that the stink bugs *A. hilare* and *M. histrionica* were differentially affected by surface sterilization of the egg masses, suggesting that various levels of mutualism occur in pentatomid-gut bacteria symbiosis. In *A. hilare*, the absence of the gut symbiont negatively impacted nymphal development, survivorship, and reproduction. In addition, mortality in surface sterilized *A. hilare* was higher mainly during the first and second instars, and few individuals reached adulthood. This explains the low R_0, T, r, λ, DT, and GRR. The high mortality suggests that the symbiont may play an important physiological role, e.g., providing *A. hilare* with essential nutrients (Abe et al., 1995; Fukatsu &

Table 3 Effect of surface sterilization on oviposition parameters in *Acrosternum hilare* (median ± 95% confidence interval; n = 11) and *Murgantia histrionica* (median ± SE). G1, generation 1; G2, generation 2

Species	Treatment/generation	nc/nf[1]	Adult emergence (days)	Pre-oviposition period (days)	Oviposition period (days)	No. eggs per egg mass
A. hilare	Control/G1	11/38	54 ± 1.56	38 ± 4.57	86 ± 25.04	25 ± 2.92
	Surface sterilized/G1	1/1	77	120	5	8
M. histrionica[2]	Control/G1	8/16	67 ± 1.68a	27 ± 3.4a	44 ± 12.01a	11 ± 0.22a
	Control/G2	4/5	75 ± 5.56a	54 ± 7.74b	26 ± 4.27a	9 ± 1.04a
	Surface sterilized/G1	6/14	69 ± 1.28a	33 ± 4.03a	53 ± 14.93a	10 ± 0.56a
	Surface sterilized/G2	0/0	–[3]	–	–	–
	$F_{2,15}$		2.02	7.98	0.84	2.71
	P		0.167	0.004	0.451	0.099

[1]Number of cohorts used for the calculation of the parameters/total number of females by the time of the first oviposition in the replicates.
[2]Means for *M. histrionica* followed by different letters within a column are significantly different (one-away ANOVA and pairwise t-tests for post-hoc comparisons).
[3]No data available.

Hosokawa, 2002). The observation that surface sterilization did not affect mortality in our preliminary trial, may have been due to the small sample size compared to the main study. Surface sterilization of *M. histrionica* eggs and consequent deprivation of its gut-associated symbiont did not show any clear effect on nymphal developmental time, and it caused a marginal increase in host survival in the first generation. No significant effect of surface sterilization on *M. histrionica*'s demographic parameters was detected, apart from the longer mean generation time in the first generation.

The symbiont infection rate in the control insects was lower for *M. histrionica* in the first (~80%) and second (~60%) generation than for *A. hilare* (~100%), and the surface sterilization method was less effective for *M. histrionica*. The technique applied, without further optimization for *M. histrionica*, was probably not sufficient to clear all symbionts from the egg masses. The detection of positive *M. histrionica* in the surface sterilization treatment likely influenced the interpretation of the results and limited our conclusions. The fact that no demographic data were collected for the second generation of *M. histrionica* in the surface sterilization treatment suggests that this species is affected by absence of the symbiont. We previously showed that *N. viridula*'s biology is unaffected by deprivation of its gut symbiont under similar lab conditions (Prado et al., 2006, 2009). However, Abe et al. (1995) showed that sterilization of egg masses negatively affected *P. stali*. Taken together, our results are consistent with previous literature reports and suggest that species in the family Pentatomidae may have a unique relationship with their respective gut-associated symbiont. Thus, the dependence of stink bug hosts on their respective gut symbionts seems to vary, at least under laboratory conditions. *Acrosternum hilare* and *P. stali* require their symbionts for survival (also Abe et al., 1995), *M. histrionica*, although not irrefutably proven, seems to have an intermediate or facultative relationship, whereas *N. viridula* seems to not require its symbiont to survive (Prado et al., 2006, 2009). The differences in host development in response to the absence of symbionts may be explained by the polyphyletic origin of the bacteria associated with the Pentatomidae (Prado & Almeida, 2009). This association is based on a unique symbiont transmission mode, which includes a short phase in which the bacteria are outside their host. The fact that the symbionts may not be obligatory for the host's survival and development does not necessarily mean that they are unimportant. There is no information on the potential role of pentatomid gut symbionts, but for the gut symbiont of a shield bug a nutritional role has been demonstrated, as well as an effect on host diapause survival (Kashima et al., 2006). It is also possible that caecal symbionts provide protection from pathogens or environmental stress.

Several papers discuss the life cycle of *M. histrionica* and *A. hilare* (Streams & Pimentel, 1963; Canerday, 1965; Simmons & Yeargan, 1988; Ludwig & Kok, 2001; Zahn et al., 2008). There are some disparities in development and demographic parameters compared to our results, probably due to different experimental methods, including the intervals the insects where censused at and/or the diets used. For example, we fed *A. hilare* green beans and peanuts, rich in fat and proteins. However, we fed *M. histrionica* broccoli heads, which are high in water content but poor in nutrients compared to beans and peanuts. Diet affects stink bug development (Todd, 1989; Zahn et al., 2008). We believe that diet played a negligible role in our study, as crucifer foliage and floral heads are used by *M. histrionica*, whereas developing fruits/seeds are dietary components for *A. hilare*, and those were included as dietary components for the insects. The slower development observed in our study may also reflect a deleterious effect of colony inbreeding, because the insects were reared for more than 6 successive generations without addition of new genotypes to the colonies (Harris & Todd, 1980, 1981). Considering the polyphagous nature of pentatomids, the diet used in future experiments should better mimic the complex nutritional environment typically exploited by the insects.

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