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Planthopper “adaptation” to resistant rice varieties: Changes in amino acid composition over time

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ABSTRACT

The brown planthopper, *Nilaparvata lugens*, shows considerable geographic and temporal variability in its response to varieties of cultivated rice. *N. lugens* has repeatedly “adapted” to resistant rice varieties; however, the physiological changes underlying planthopper adaptation are poorly understood. Endosymbionts within planthoppers, such as yeast-like endosymbionts (YLS) could play a role as they produce essential amino acids for planthoppers. We used a full factorial study to determine how natal rice variety, exposed rice variety, YLS presence, and the number of reared generations affected nymphal development, planthopper total nitrogen content, and planthopper hydrolyzed amino acid profiles. Nymphal development was strongly influenced by a four-way interaction between the exposed rice variety, natal rice variety, number of reared generations, and YLS presence. While symbiosis improved nymphal performance in the 8th generation, it appeared to be a drain on nymphs in the 11th generation, when the aposymbiotic nymphs actually showed higher performance than the symbiotic nymphs. This suggests that the symbiotic relationship may be acting beneficially in one generation while acting as a drain during another generation. Aposymbiotic planthoppers reared for 11 generations had a higher proportional concentration of rare amino acids than those reared for 8 generations, indicating that the planthopper itself appears to improve its ability to acquire rare amino acids. Therefore, the change in amino acid composition of planthoppers suggests an underlying change in protein expression or amino acid metabolism over time.

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1. Introduction

The brown planthopper, *Nilaparvata lugens* Stål (Hemiptera: Delphacidae), shows considerable geographic and temporal variability in its response to cultivated rice varieties (Heinrichs et al., 1985; Pathak and Khush, 1979; Pathak and Heinrichs, 1982). As a product of the Green Revolution (Kenmore et al., 1984), *N. lugens* outbreaks have threatened rice production and food security (Cohen et al., 1997; Dyck and Thomas, 1979). Although there has been significant investment in breeding for rice resistance to the brown planthopper in Asia (Cha et al., 2008; Cohen et al., 1997; Heinrichs et al., 1985; Jairin et al., 2007; Jena et al., 2006; Lu et al., 2007; Park et al., 2007; Su et al., 2006; Sun et al., 2007), the emphasis on gene discovery for planthopper resistance has severe shortcomings. *N. lugens* has repeatedly shown the ability to rapidly “adapt” to resistant rice varieties after several generations of continuous rearing in

the field and laboratory (Claridge and den Hollander, 1983; Claridge et al., 1982, 1984; Gallagher et al., 1994). We use the term “adaptation” to describe how planthoppers increase their performance on a single rice variety over several generations. Given that planthopper “adaptation” occurs over months, it appears to occur too rapidly to result from selection. Before lasting progress can be made in breeding for rice resistance, it is important to determine what factors underlie planthopper “adaptation” to resistant rice varieties.

Planthopper “adaptation” to resistant varieties can be measured through increases in survival, body weight, honeydew production, and/or reproductive fitness (Pathak and Heinrichs, 1982). Host plant nutrition is thought to be more important than defenses, because delphacid planthoppers almost exclusively feed on monocots, which contain lower levels of plant allelochemicals (Harbone and Williams, 1976; Prestidge and McNeill, 1983). Resistant rice varieties generally have higher levels of phenolic compounds, lower levels of free amino acids, and lower levels of reducing sugars (Das, 1976; Grayer et al., 1994; Mishra et al., 1990; Thayumanavan et al., 1990). Variation in amino acid abundance and composition may affect planthopper fitness and devel-

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opment as well. On nitrogen-deficient rice plants, *N. lugens* switch their feeding sites more often, and as a result, excrete less honeydew (Sogawa, 1982). Also, varieties with lower asparagine content are considered to be resistant to planthoppers (Chino et al., 1987; Sogawa and Pathak, 1970).

Microbial symbiosis has been linked with insect groups that specialize in feeding on plant phloem (Douglas, 1998). These symbiotic microorganisms improve diet quality by synthesizing essential amino acids lacking in the insect's diet (Douglas, 1989, 1998). In this study, we denote "essential" amino acids as those amino acids found in planthoppers, but rare in the planthopper's diet, which consists mostly of the amino acids asparagine/aspartate and glutamine/glutamate. While aphid symbiosis has been studied in great detail, much less is known about planthopper endosymbionts and their role in planthopper metabolism (Wilkinson and Ishikawa, 2001). In planthoppers, yeast-like endosymbionts (YLS) reside intracellularly in the planthopper's fat body cells (Buchner, 1965; Cheng and Hou, 1996; Noda, 1974). YLS provide rare nutrients to the planthoppers to compensate for the unbalanced composition of amino acids in plant phloem (Noda and Saito, 1977, 1979). When YLS are experimentally removed, *N. lugens* nymphs lose weight and grow more slowly (Wilkinson and Ishikawa, 2001). Without YLS, planthoppers have lower total protein concentrations, higher levels of non-limiting free amino acids such as glutamine and aspartate, and significantly lower levels of other amino acids such as leucine (Wilkinson and Ishikawa, 2001). It is difficult to speculate on how reliant *N. lugens* are on YLS because artificial rearing experiments have shown that no single amino acid is considered limiting for *N. lugens* (Koyama, 1985). Experimental results confirm that YLS play a role in amino acid metabolism through the recycling of uric acid (Sasaki et al., 1996). Therefore, YLS appear to support both planthopper nutrition and development.

N. lugens reared continuously in culture appear to physiologically specialize on the rice variety that they are reared upon within several generations, but show a reduced performance on other varieties (Claridge and den Hollander, 1980; Claridge and Den Hollander, 1982; Saxena and Barrion, 1983). These selected populations have been named "biotypes" in other studies, but the use of this term is problematic. The rapid "adaptation" of *N. lugens* on reared rice varieties strongly suggests that the "biotypes" appear to be selected populations rather than genetically distinct and diverging host races (Claridge and den Hollander, 1983; Shufran and Whalon, 1995). However, other biological factors could contribute to these patterns of "adaptation". When transferred from a susceptible rice variety to resistant rice varieties (ASD7 or Mudgo), Lu et al. (2004) found that planthoppers showed a decrease in nymphal performance and survival in the first generation, coupled with a decrease in YLS densities and transaminase activity. In the subsequent 2nd and 3rd generations, nymphal performance improved and YLS became more abundant. As a result, Lu et al. (2004) suggested that YLS may be linked to variation in planthopper performance and adaptation to rice resistance.

In this study, we examined if planthopper amino acid composition and development was influenced by the natal rice variety, the exposed plant variety, YLS presence, or the number of generations associated with a particular variety. We used a full factorial design to assess the relationship between the number of generations reared on a host plant, presence of YLS, natal plant variety, and exposed plant variety on planthopper amino acid composition and performance. We assessed planthopper nutrition by examining planthopper total nitrogen content and hydrolyzed amino acid profiles. Specifically, we used the full factorial design to ask: (1) How does continuous exposure on one rice variety lead to trade-offs in performance and amino acid composition (nymphal development, planthopper total nitrogen content, and proportional levels of hydrolyzed amino acids) on another rice variety? (2) How

does the presence of YLS influence planthopper metabolic trade-offs on different rice varieties? 3) How does the natal host plant, exposed host plant, number of generations interact to influence planthopper amino acid composition and performance?

2. Methods

2.1. Rearing of study organisms

We used three rice varieties Taichung Native 1 (TN1), Mudgo, and ASD7 that vary in their resistance and have been extensively used in studies of planthopper adaptation to resistant rice varieties (Claridge and den Hollander, 1980; Claridge and Den Hollander, 1982; Saxena and Barrion, 1983; Saxena and Pathak, 1979). TN1 is considered to be susceptible to *N. lugens*, while Mudgo is thought to carry the *Bph 1* gene for resistance and ASD7 is thought to carry *Bph 2* for resistance (Heinrichs et al., 1985). Susceptible varieties tend to have higher levels of free amino acids than resistant rice varieties (Sogawa, 1982; Thayumanavan et al., 1990). The rice varieties chosen for this study also significantly differ in amino acid content, with Mudgo and ASD7 showing lower levels of free amino acids than susceptible varieties such as TN1 (Saxena, 1986; Sogawa and Pathak, 1970).

A colony of *N. lugens* has been continuously reared in the greenhouse since the early 1960's on the susceptible variety, Taichung Native 1 (TN1), at the International Rice Research Institute (IRRI) in the Philippines. Since 2004, field-collected planthoppers have been added annually to prevent excessive inbreeding. Using the TN1 colony, we establish three colonies on the rice varieties TN1, Mudgo, and ASD7 using an initial population of 500 planthopper adults. Planthoppers were reared on TN1, Mudgo, and ASD7 for 5 generations as three separate colonies before the start of the experiment.

TN1, Mudgo, and ASD7 plants were raised by sowing seeds into plastic trays (51 × 39 × 10 cm) half-filled with field-collected top soil. Seven days after sowing, a single rice seedling was transplanted into a circular clay pot (7 cm diameter × 12 cm depth). Plants were fertilized at a rate consistent with field practices (150-60-20 NPK). The pots were held in galvanized iron trays filled with water to simulate flooded conditions. The plants were reared for 10 days in the greenhouse before use in the study.

2.2. Treatments

We raised plants in the glasshouse at the International Rice Research Institute. The experimental study was conducted in growth chambers (Thermo Scientific) at 25 °C under a 12:12 L:D light regime. We used a full factorial design to assess the role of the natal rice variety, exposed rice variety, YLS presence, and the number of reared generations on nymphal development, total nitrogen within the planthopper, and hydrolyzed amino acid profiles of the planthopper. The treatments consisted of three natal rice varieties (TN1, Mudgo, and ASD7), three exposed rice varieties (TN1, Mudgo, and ASD7), symbiosis (planthoppers with normal and reduced YLS abundance), and the number of generations (6th and 8th generation). The term "natal rice variety" refers to the planthopper culture that was started from a TN1 planthopper colony and reared on a particular variety throughout the duration of the study. For each assayed generation, planthoppers were taken each planthopper colony and exposed to one of the three rice varieties. Therefore, the full factorial study consisted of a total of 36 treatment combinations.

While the yeast-like endosymbionts cannot be completely eliminated like aphid bacterial endosymbionts, exposure to high temperatures can largely remove the endosymbionts without

negatively impacting the planthoppers (Noda, 1974; Wilkinson and Ishikawa, 2001). YLS are associated with planthoppers at every stage of development, and are YLS are transmitted to the subsequent generation through transovarial transmission. While the heat treatment does not completely remove the YLS, heat-treated planthoppers contain 5% of the YLS abundance levels of untreated planthoppers (Chen et al., 1981). Given that YLS could not be removed completely, the YLS removal treatment was conducted separately for each generation studied. In this study, we use the term “aposymbiotic” to denote planthoppers that have been exposed to the heat treatment for three days after hatching.

Following Noda and Saito (1979), YLS-depleted insects were generated by rearing newly-hatched planthoppers on the three treatment varieties at 35 °C for 3 days in a growth chamber, and all symbiotic and YLS-depleted planthoppers were held at 25 °C (12:12 L:D) for the remaining 5 days. In order to quantify whether the heat treatments reduced YLS abundance, planthoppers from each of plant were immediately frozen at –80 °C. Each replicate consisted of six planthoppers ground up in 200 µl of saline solution, and 16 replicates were completed for each rice variety. A 2 µl drop of the planthopper suspension was added to a hemocytometer, and the number of YLS was counted under a compound microscope. Each cell was equivalent to 0.1 µl. We tested if the heat treatment or rice variety influenced YLS abundance in two-way ANOVA. While the rice variety did not influence YLS abundance, we observed that the heat treatment resulted in a ~90% reduction in YLS abundance (1688.95 ± 85.01 YLS/µl vs. 168.33 ± 10.98 /µl; $t = 17.74$, $df = 94$, $P < 0.0001$).

2.3. Planthopper development

Ten newly-emerged nymphs were placed onto each of six treatment plants (TN1, Mudgo, and ASD7), for a starting population of 60 nymphs per treatment. Symbiotic planthoppers were held on the treatment plants at 25 °C, while YLS-depleted planthoppers were created by holding planthoppers on treatment plants at 35 °C for 3 days. After the heat exposure, all plants were held at 25 °C for an additional 5 days. Surviving planthoppers were weighed individually on a microbalance with a sensitivity of 0.01 mg (Sartorius ME0215S). The procedure was repeated using planthoppers collected from the 8th and 11th generations.

2.4. Total nitrogen analysis

We tested if total nitrogen varied among the planthoppers from the different treatments. We placed 50 *N. lugens* nymphs on each of 25 treatment plants, following the full factorial design (3 natal varieties × 3 exposed varieties × YLS presence), for a total of 18 treatment combinations. Due to the high number of individuals (22,500) needed for each replicate; we completed three replicates of the full factorial design temporally. Therefore, each replicate consisted of a separate planthopper generation. Nymphs were reared for 8 days, and surviving insects were dried at 80 °C for 24 h, weighed (Sartorius BP110S, 0.1 mg sensitivity), and pooled to generate enough sample for the analysis. A minimum of 5 mg of dried planthopper material was required by the Analytical Service Laboratory (ASL) at IRRRI for the total nitrogen analysis, which ranged from 165 to 996 individuals for each treatment. Dried planthoppers were finely ground, and 2.5 mg were used to quantify total N on a Thermo Scientific FlashEA 1112 nitrogen and carbon analyzer, previously calibrated using known standards.

2.5. Planthopper amino acid analysis

The hydrolyzed amino acid content of the planthoppers was determined using reverse-phase high performance liquid chroma-

tography (HPLC). In order to standardize nymphal age, 100 gravid female adults were introduced into a cage with TN1, Mudgo, and ASD7 plants to lay eggs for 12 h. We placed five newly-emerged planthopper nymphs onto individual 30 day-old TN1, Mudgo, or ASD7 plants, a density lower than the economic injury level (Heinrichs et al., 1985). The number of treatment plants varied because the number of planthopper nymphs needed for 2 mg of sample varied. Planthoppers were randomly designated for the YLS-removal treatments. After exposure to heat treatment for 3 days, all treatment plants were placed in a growth chamber at 25 °C for an additional 5 days, after which surviving insects were immediately frozen at –80 °C. The study was repeated using the 6th and 8th planthopper colony generations.

Each replicate consisted of 2 mg of frozen planthoppers collected from each treatment. The number of planthoppers required ranged from 8 to 16 individuals per replicate. Planthoppers were homogenized with 100 µl ice-cold 80% methanol, and the homogenate was centrifuged for 20 min at 15000g. The supernatant was removed, and the remaining sample was vacuum-dried, hydrolyzed with 100 ml of constant boiling hydrochloric acid and 1 mg phenol. The insect protein were transferred into borosilicate V-vials, purged of oxygen with nitrogen gas and sealed immediately by lining the vials with Teflon tape before replacing the cap. The sample was dried in an oven at 110 °C for 20 h. Nanopore water was added to the hydrolysate to reach 1 ml, and then passed through a 0.22 µ filter.

Twenty microliter of the filtered hydrolysate was vacuum-dried, then reconstituted with 10 µl of 20 mM constant boiling HCl. The reconstituted sample was derivatized with the AccQ-Fluor reagent kit (WAT052880-Waters Corporation, Milton, MA, USA). Using a micropipetter, 70 µl of AccQ-Fluor borate buffer was added in the sample tube and vortexed briefly. We then added 20 µl of AccQ-Fluor reagent and vortexed the sample immediately for several seconds. After 1 min, the contents were transferred to an auto-sampler vial with a low volume insert and capped with a silicone-lined septum. The vial was heated for 10 min in a waterbath at 55 °C before HPLC separation.

The AccQ-Fluor amino acid derivatives were separated on a Waters 2695 Separations Module HPLC System attached to a Waters 2996 Photodiode Array Detector. A 5 µl sample was injected into a Waters Nova-Pak C₁₈ Silica-bonded Column (150 mm × 3.9 mm). The Waters AccQ Tag Eluent A Concentrate was used as eluent A (WAT052890) and 60% acetonitrile as eluent B in a separation gradient (Appendix).

The amino acids were detected using a Waters Photodiode Array Detector (Model PDA 2996, Waters Corporation) with the column condition set at 37 °C. The amino acid peaks were acquired using Empower Photodiode Array (PDA) software by Waters Corporation (2002) and were calculated based on an amino acid calibration standard (PIERCE Amino Acid Standard H, Product No. 20088) run at three concentrations (10, 20, 40 µM). Amino acid assignments were visually checked to verify the peak assignment. For the amino acid analysis, we calculated the molar concentration of the sample, and the proportional molar concentration for each amino acid. Sample quantities were converted from pmol/µl to nmol/ml, and then divided by the number of insects found in each sample. Therefore, the data are shown as the average hydrolyzed molar concentration of the amino acids per insect.

2.6. Statistical analysis

We used a mixed-effects ANOVA to test the effect of the natal host plant, exposed plant, generation, YLS presence and all possible interactions on planthopper nymphal weights in JMP 7.0.1 (SAS Institute, Cary, NC). We designated the natal and exposed host plant treatments as fixed effects, and generation and YLS presence

as random effects. We used a stepwise process to eliminate the non-significant factors and determine the influence on the overall fit of the model. For factors that were significant, a post hoc Tukey's HSD test was applied to determine which treatments were significantly different from each other.

In order to determine if hydrolyzed amino acid concentrations differed among the treatments, we tested for the effect of natal rice variety, exposed rice variety, endosymbiont presence, and generation on the abundance of the 16 amino acids using a MANOVA test using the function PROC GLM in SAS 9.1. We tested the significance of all possible interactions and main effects using a stepwise regression approach. Because the amino acids did not uniformly respond to the treatment factors, we performed further ANOVA tests to determine how each amino acid differed between factors found significant in the MANOVA. The significance value of the tests was adjusted using a Bonferroni correction. A Tukey's HSD was used for pairwise comparisons for amino acids that showed significant ANOVA differences.

3. Results

3.1. Planthopper performance

The mixed-model ANOVA showed a four-way interaction between planthopper generation, natal host plant, exposed host plant, and YLS presence was significant in explaining variation in nymphal weight (Table 1). The four-way interaction could be best explained by an increase in aposymbiotic nymphal performance on their natal resistant varieties (ASD7 and Mudgo) over time (Fig. 1). Aposymbiotic nymphal weights showed greater variation among the rice varieties initially, and aposymbiotic nymphal weights showed a more dramatic increase on resistant varieties (ASD7 and Mudgo) from the 8th generation to the 11th generation (Fig. 1). However, the same patterns were not observed in symbiotic planthoppers. There was also a strong three-way interaction between generation, natal host plant and exposed host plant (Ta-

Table 1

Results from an ANOVA testing for the effects of the natal host plant variety (TN1, Mudgo, and ASD7), exposed host plant (TN1, Mudgo, and ASD7), and YLS presence (symbiotic or aposymbiotic), and the number of reared generations (8th or 11th) on *N. lugens* nymphal weights after 8 days of feeding.

Source	df	F ratio	P
Generation	1, 1824	7.02	<0.01
Natal host plant	2, 1824	1.05	NS
YLS presence	1, 1824	13.96	<0.001
Exposed host plant	2, 1824	11.49	<0.0001
Generation × natal host plant	2, 1824	0.38	NS
Generation × YLS presence	1, 1824	47.23	<0.0001
Generation × exposed host plant	2, 1824	2.64	NS
Natal host plant × exposed host plant	4, 1824	1.77	NS
Natal host plant × YLS presence	2, 1824	0.02	NS
Exposed host plant × YLS presence	2, 1824	10.54	<0.0001
Generation × natal host plant × exposed host plant	4, 1824	5.43	<0.001
Generation × natal host plant × YLS presence	2, 1824	0.12	NS
Generation × exposed host plant × YLS presence	2, 1824	1.86	NS
Natal host plant × exposed host plant × YLS presence	4, 1824	0.43	NS
Generation × natal host plant × exposed host plant × YLS presence	4, 1824	3.22	<0.05

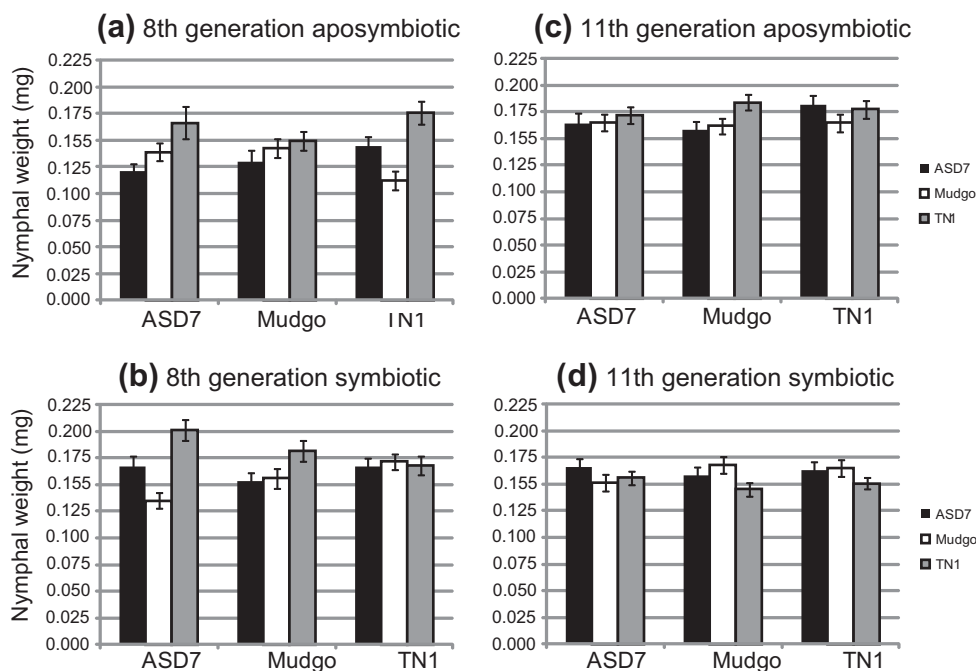


Fig. 1. Planthopper nymphal weights after 8 days of feeding: (a) 8th generation aposymbiotic planthoppers, (b) 8th generation symbiotic planthoppers, (c) 11th generation aposymbiotic planthoppers, and (d) 11th generation symbiotic planthoppers. Clustered columns represent planthoppers reared on the same host plant, while the columns represent planthoppers on the exposed rice variety.

Table 2

Results of MANOVA testing the effects of the number of planthopper generations, YLS presence (symbiotic or aposymbiotic), the natal host plant variety, exposed host plant variety, and an interaction between the number of generations and YLS presence on (a) amino acid total concentrations and (b) the proportional composition of amino acids.

Factor	Wilks' lambda	F	Num DF	Den DF	P
<i>(a) Amino acid concentrations</i>					
Generation	0.26	44.97	17	264	<0.0001
YLS presence	0.29	38.08	17	264	<0.0001
Natal host plant	0.93	0.55	34	528	NS
Exposed host plant	0.74	2.52	34	528	<0.0001
Generation × YLS presence	0.77	4.59	17	264	<0.0001
<i>(b) Proportional composition of amino acids</i>					
Generation	0.24	53.48	16	265	<0.0001
YLS presence	0.38	26.62	16	265	<0.0001
Natal host plant	0.93	0.58	32	530	NS
Exposed host plant	0.83	1.64	32	530	<0.05
Generation × YLS presence	0.82	3.68	16	265	<0.0001

ble 1). In general, all planthoppers, regardless of their natal plant variety tended to perform better on TN1. This pattern confirmed previous findings that nymphs appear to show increased performance on one variety, resulting in a decreased performance on other varieties (Fig. 1). However, this pattern was much stronger among the aposymbiotic planthoppers than the symbiotic planthoppers (Fig. 1).

There were two significant two-way interaction effects influencing nymphal weight. The exposed host plant and YLS presence showed an interaction in their effect on nymphal weights. Symbiosis had a stronger positive effect on nymphal weights reared on resistant varieties (ASD7 and Mudgo) than on TN1 (Fig. 1a–d). The number of reared generations and YLS presence showed a interaction in influencing nymphal development. While symbiosis increased nymphal weights in the 8th generation, it did not clearly increase nymphal weights in the 11th generation. In fact, symbiosis strongly decreased nymphal weight on TN1 in the 11th generation.

3.2. Total nitrogen content

The mixed effects ANOVA model showed that planthopper total percent nitrogen was influenced by YLS presence ($F_{1,48} = 66.73$, $P < 0.0001$), the experimental replicate ($F_{2,48} = 9.73$, $P < 0.001$), and an interaction between experimental replicate and YLS presence ($F_{2,48} = 15.52$, $P < 0.0001$). The natal host plant variety and exposed host plant did not influence planthopper total percent nitrogen. Total percent nitrogen was, however, significantly enhanced in symbiotic over aposymbiotic planthoppers (9.93 ± 0.18 mg vs. 8.68 ± 0.10 mg; Table 2). The interaction between the experimental replicate and endosymbiont presence was due to an increase in total percent nitrogen over the temporal replicates in symbiotic planthoppers, but a decrease in asymbiotic planthoppers (Fig. 2).

3.3. Planthopper amino acid content

The MANOVA models examined the role of the natal host plant, exposed host plant, YLS presence, the number of generations, and all possible interactions on the abundance of amino acids and the proportional abundance of individual amino acids. Both MANOVA models showed similar results, the four-way interaction and all three-way interactions were not significant (Table 2a and b). The exposed rice variety, YLS presence, the number of generations reared on a rice variety, and an interaction between the number of generations and YLS presence were the strongest factors explaining variation in amino acid concentrations per insect. While total percent nitrogen differed between symbiotic and aposymbi-

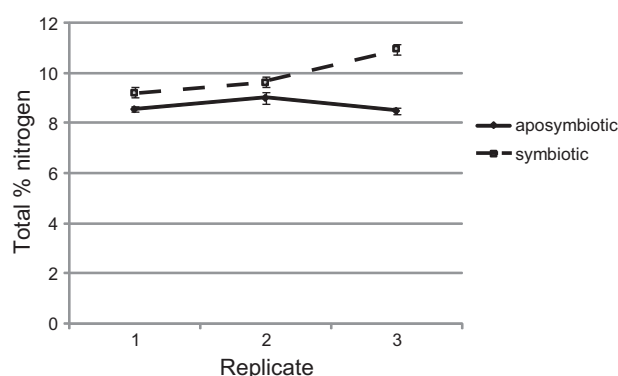


Fig. 2. Total percent nitrogen of aposymbiotic and symbiotic planthoppers over the three experimental replicates.

otic planthoppers, the significance of these other factors indicated that planthoppers differed significantly in their amino acid composition.

If planthoppers were most physiologically specialized on their maternal host plant, there should have been an interaction between the natal plant and exposed plant on amino acid concentrations. This pattern was not supported by the hydrolyzed amino acid profiles (Table 2a and b). Amino acid concentrations and the proportional composition of amino acids were significantly influenced by the exposed rice variety, but not the natal variety (Table 2a and b and Fig. 3a). All amino acids were significantly higher on TN1 than on ASD7 or Mudgo (Fig. 3a). While the absolute concentrations of amino acids varied among planthoppers reared on different rice varieties (Table 2a), only the proportional concentration of aspartate, alanine, and leucine were different (Fig. 3b). Aspartate concentrations were proportionally highest in planthoppers that fed on TN1, and lowest on Mudgo (Fig. 3b). Alanine concentrations were similar between ASD7 and Mudgo, but lower for planthoppers reared on TN1. The proportional concentration of leucine was highest for Mudgo and lowest on TN1 (Fig. 3b).

YLS presence significantly increased the proportion of amino acids. There was also an interaction between planthopper generation and YLS presence in explaining variation in amino acid concentrations and the proportional composition of amino acids (Table 2, Fig. 4a). Planthoppers from the later generation and aposymbiotic planthoppers showed decreased aspartate, glutamate, and methionine concentrations. As a consequence, 8th generation planthoppers with YLS had the highest proportions of serine, glycine, histidine, arginine, alanine, proline, tyrosine, isoleucine, leucine, and phenylalanine (Fig. 4b).

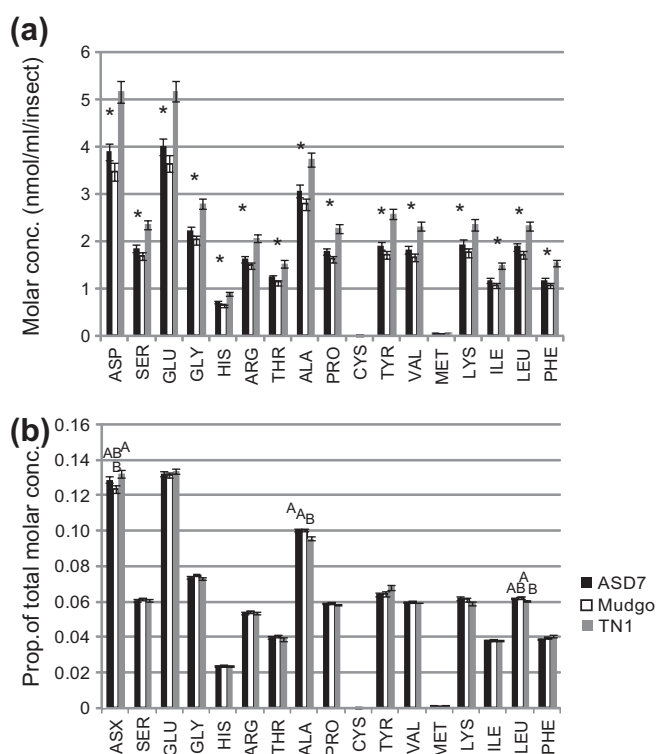


Fig. 3. Amino acid (a) molar concentrations and (b) proportion of total molar concentration for planthoppers exposed to the rice varieties ASD7, Mudgo, and TN1. Asterisks (*) and letters represent concentrations that were significantly different following Tukey's HSD test ($P < 0.05$).

The number of reared generations significantly altered the proportional amino acid content; the later generation showed a higher proportion of rare amino acids (Table 2; Fig. 4b). The proportions of serine, glycine, histidine, proline, tyrosine, isoleucine, leucine, and phenylalanine increased in the later planthopper generation, while the proportional concentrations of aspartate, glutamate, alanine, valine, methionine, and lysine decreased. YLS presence also interacted with some of the amino acids; the proportional abundance of tyrosine was highest in the later generation or planthoppers without YLS. Also, the proportional content of lysine was highest for 6th generation symbiotic planthoppers and lowest in 8th generation aposymbiotic planthoppers. Extended rearing and YLS presence lowered the proportional molar concentration of the amino acids, aspartate and glutamate. Therefore, 8th generation planthoppers had the lowest proportional levels of aspartate and glutamate and higher levels of rarer amino acids. Therefore, extended rearing appeared to increase planthopper conversion of non-limiting amino acids to rarer amino acids (Fig. 4b).

The presence of YLS in symbiotic planthoppers significantly enhanced the proportion of most of the amino acids (Fig. 4b): serine, glycine, arginine, threonine, alanine, tyrosine, valine, lysine, isoleucine, and leucine. Only proportional levels of aspartate and glutamate levels were significantly higher in aposymbiotic planthoppers (Fig. 4b). While YLS presence increased the concentrations of rare amino acids, the study did not detect an interaction between YLS presence and either the exposed or natal host plant. Therefore, the exposed host plant and YLS showed independent effects on planthopper amino acid content (Table 2).

4. Discussion

This study found that *N. lugens* appear to change in their amino acid composition on different rice varieties after several genera-

tions of continuous rearing. The pattern was consistent regardless of the exposed rice variety. Extended rearing increased the total nitrogen content and proportional concentrations of rare amino acids, while decreasing the proportion of common amino acids such as aspartate and glutamate. While continuous exposure to one variety led to trade-offs in nymphal performance on other varieties, we did not detect that planthoppers became more specialized on a single variety, resulting in trade-offs in amino acid composition on other varieties. Therefore in relation to our first question, we found that continuous rearing on the same variety led to an interaction in nymphal performance on other varieties, but natal variety, exposed variety and the number of generations did not interact to influence hydrolyzed amino acid content. Our findings indicate that planthoppers from different generations and raised on different varieties had a different amino acid composition, indicating that they were structurally different. These results are unexpected given that hydrolyzed amino acid concentrations are the product of gene translation and transcription, processes previously thought to be relatively consistent within species (Sandstrom and Moran, 1999). Also, insects are thought to compensate for poor diet quality by increasing the rate and quantity of food intake (Chapman, 1998), so metabolic pathways might adjust to low quality foods that have low concentrations of essential nutrients. Therefore, planthoppers raised under the different treatment conditions could have been structured differently.

The significant variation in hydrolyzed amino acid profiles showed that amino acid composition in planthoppers can be quite plastic. There are several factors that could help to explain these results. Variation in planthopper size and allometry could have amplified the amino acid concentrations when multiple individuals were pooled together for the analysis. If particular amino acid concentrations were correlated with organ size, then variation in planthopper size alone would have caused variation in amino acid concentrations. In that case, variation in nymphal size among rice varieties would have been correlated with different absolute and proportional amino acid concentrations. The lack of a relationship between these variables indicates that the variation in amino acid concentrations was a consequence of different growth on the different treatments (Figs. 1 and 3). Furthermore, the results were standardized by the number of insects in the sample, which would account for any inflation in variation. Another possibility is that the differences in amino acid concentrations were that they were the product of shifts in types or amount of expressed protein. Host plant resistance or the nutritional composition in an insect diet can alter patterns of gene expression (Freitak et al., 2009; Yang et al., 2005, 2006). If the dominant proteins expressed differed among the planthopper treatments, changes in protein expression could have altered the proportional levels of the amino acids.

Researchers have observed that planthoppers appear to adapt to new rice varieties through significant increases in nymphal weight and honeydew production over several generations (Claridge and Den Hollander, 1982; Gallagher et al., 1994; Sogawa, 1982). Because YLS plays an important role in amino acid metabolic efficiency (Wilkinson and Ishikawa, 2001), we hypothesized that YLS could be related to nymphal performance and adaptation to different rice varieties. The first question asked how continuous exposure on once rice variety influenced trade-offs in performance and amino acid composition. The interaction between planthopper generation, YLS presence, exposed rice variety, and natal rice variety on nymphal performance supports the notion that planthoppers appear to specialize on the natal rice variety, which may lead to tradeoffs with other varieties. However, we did not find clear consistent pattern in terms of the importance of certain factors on nymphal performance. In general, all planthopper nymphs performed relatively well on the susceptible variety TN1. Previous nymphal performance studies have found that enhanced perfor-

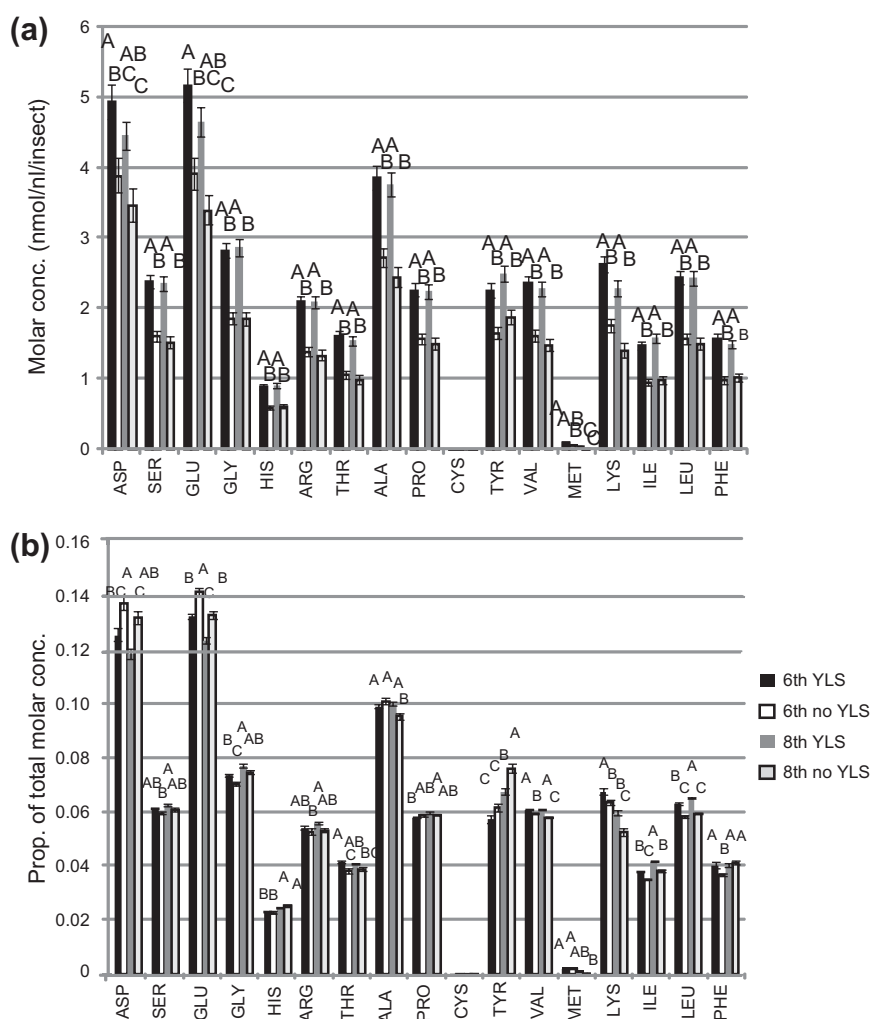


Fig. 4. Amino acid (a) molar concentrations and (b) proportion of total molar concentration for 6th and 8th generation symbiotic and asymbiotic planthoppers. Letters above the columns represent concentrations that were significantly differently following Tukey's HSD test ($P < 0.05$).

mance on one variety sometimes but not always results in a trade-off on another variety (Roderick, 1994). In this study, we failed to detect an interaction between the natal and exposed host plant varieties on the hydrolyzed amino acid profiles, as amino acid content was most strongly determined by the exposed host plant. Planthoppers exposed to TN1 had the highest concentrations of all of the major amino acids. The only trade-off in performance was for planthoppers transferred from TN1 to the resistant variety Mudgo (Fig. 1). The proportional concentration of aspartate, alanine, and leucine differed among planthoppers exposed to different rice varieties, but did not follow a consistent pattern.

Our second major question was to determine how the presence of YLS influence planthopper metabolic trade-offs on different rice varieties. We did not find any support that the presence of YLS significantly interacted with the exposed or natal plant varieties (Table 2). We also asked how the natal host plant, exposed host plant, number of generations interacted to influence planthopper amino acid composition and performance. We found that there was an interaction between the number of generations and YLS presence on amino acid composition. Unexpectedly, we found significant increases in nymphal weight with extended rearing on the same variety, especially for aposymbiotic planthoppers. YLS removal during the later generation substantially increased nymphal performance, suggesting that YLS could possibly act as a metabolic drain on the planthopper. However, this study only examined nymphal performance and amino acid composition over 2 genera-

tions, so YLS could play a role in improving nymphal performance during the early generations after initial exposure to a new variety, as suggested by Lu et al. (2004).

While we did not detect a metabolic trade-off in this study for hydrolyzed amino acid concentrations, metabolic trade-offs may be better assessed using other assays. Free amino acid concentrations may be more reflective of metabolic trade-offs on different host varieties, as they reflect short-term stoichiometric limitations. In a recent study, Xu et al. (2008) found that *N. lugens* transferred from TN1 to the resistant variety IR26 resulted in elevated free amino acids (aspartate and glutamate) in planthopper tissues and honeydew during the first generation. However, free amino acid levels then decreased by the third generation, indicating an increase in planthopper metabolic efficiency. Despite similar levels of free amino acids in the phloem between IR26 and TN1, the work by Xu et al. (2008) suggests that planthoppers could be less efficient in protein anabolism when initially exposed to new resistant varieties. Further work should continue to contrast initial and later generations of planthoppers reared on susceptible and resistant varieties.

The third major question of the study focused on determining how does the natal host plant, exposed host plant, number of generations interact to influence planthopper amino acid composition and performance. While the host plants showed influence nymphal development, they did not dramatically influence amino acid composition. However, the number of generations reared in culture

had a large effect on nymphal development and amino acid composition. An interaction between YLS presence and the experimental replicate influenced planthopper total nitrogen content. A possible explanation for this is that the third replicate was performed close to a year after the first two replicates, translating to approximately twelve more colony generations. The assay used the same artificial rearing conditions, so the only major difference between the replicates was the additional time exposed to the different varieties. It appears as if the additional time can influence the partitioning of N assimilation between the planthopper and YLS. The absence of YLS decreased total N content by about 14%, similar to previous estimates (Wilkinson and Ishikawa, 2001). While symbiotic *N. lugens* showed an increase in total percent nitrogen over the temporal replicates, aposymbiotic planthoppers showed a decrease in percent nitrogen. Combined with the nymphal performance data, these results suggest that amino acid partitioning in the YLS-planthopper relationship may be temporally dynamic.

Extended rearing and YLS presence showed an interaction on total concentrations and the proportional concentrations of several amino acids. There was a decrease in the total molar concentrations of aspartate, glutamate, and methionine between the 6th and 8th generation. Given that asparagine/aspartate and glutamate are abundant in the rice phloem (Hayashi and Chino, 1990), these changes suggest a change in either gene expression or metabolism. However, analysis of the proportion molar concentrations revealed that planthoppers reared for 8 generations showed an increase in glycine, histidine, isoleucine, leucine, and tyrosine (Fig. 4b). The only amino acid to decrease from the 6th to the 8th generation was lysine (Fig. 4b). While YLS presence clearly increased the proportion concentrations of some amino acids (glycine, threonine, alanine, valine, and lysine), the gains in the proportional amino acid concentrations for glycine, histidine, isoleucine, leucine, and tyrosine increased with the number of generations rather than YLS (Fig. 4a). Therefore, we found a interaction between the number of generations reared and YLS presence.

The presence of YLS increased the total and proportional molar concentrations of rare amino acids. While the hydrolyzed amino acids were generally higher in the presence of YLS, there were a few notable exceptions. Tyrosine levels were higher in the absence of YLS, suggesting that tyrosine may be more required by YLS metabolism than by the planthopper. Leucine concentrations increased over time in both the aposymbiotic and symbiotic planthoppers, suggesting changes within the planthopper in the production of essential amino acids. Wilkinson and Ishikawa (2001) suggested that leucine might be limiting in aposymbiotic planthoppers. In this study design, it was not possible to evaluate if there were signs of differences in leucine limitation that might led to patterns of specialization. We did not find any support for limitations in amino acid availability. Planthoppers reared on Mudgo actually had the highest levels of leucine, while planthoppers on TN1 reared had the lowest. Therefore, leucine availability did not correlate well with plant resistance.

Variation in amino acid concentrations in the phloem could have influenced planthopper development and metabolism. Amino acid content in the rice phloem within the leaf sheath is dominated by asparagine or aspartate (19.4%), serine (12.4%), glutamic acid or glutamate (13.6%), and glutamine (19.0%) (Hayashi and Chino, 1990). Noda et al. (1973) found that asparagine, glutamic acid, and valine dominated the amino acid composition of planthopper honeydew, and minimal levels of cysteine, methionine, and tryptophan were detected. By rearing *N. lugens* on different diets each lacking in one amino acid, Koyama (1985) found that the growth of *N. lugens* was strongly delayed when cysteine, histidine, and methionine were lacking in the diet. Therefore, limited availability of particular amino acids may constrain planthopper growth. Fu-

ture studies could examine how interactions among planthopper generation, natal plant variety, and exposed plant variety could affect free amino acid concentrations within the planthoppers. By directly relating planthopper free amino acid budgets with phloem free amino acid levels in different varieties, insight might be gained on the metabolic efficiency of different planthoppers on different rice varieties. The challenge for such a study design would be in acquiring samples of rice phloem. Unlike aphids, planthoppers retract their stylets when stressed, so aphid stylectomy cannot be used for phloem extraction (Kawabe et al., 1980). For rice, specialized laser equipment has been developed to perform stylectomy in order to extract rice phloem (Fukumorita and Chino, 1982; Hayashi and Chino, 1990).

While this study focused on hydrolyzed amino acid composition, clearly both nutritional and defensive factors can influence planthopper growth, gene expression, and metabolism. As a defense against herbivory, plants produce proteinase inhibitors, which bind to insect proteases and inhibit their activity. Wang et al. (2008) found that planthopper damage on a resistant rice variety induced an aspartic proteinase encoding gene, a serine protease-like protein gene, and a rice proteinaceous cysteine proteinase inhibitor gene, but the same responses were not found in a susceptible rice variety. The ingestion of proteinase inhibitors is thought to lead to amino acid deficiencies in insects (Ryan, 1990). Cysteine and serine proteases have been detected in the guts of *N. lugens*, and it is thought that these enzymes play a significant role in interfering with planthopper protein digestion (Foisac et al., 2002). The presence of proteinase inhibitors in rice and proteases in the guts of planthoppers suggest that defenses could play a significant role in contributing to patterns of amino acid utilization efficiency. Therefore, planthopper adaptation to resistant rice varieties could involve overcoming plant defenses as well.

The question remains why amino acid composition fluctuates so greatly and rapidly among planthoppers reared on the same rice variety for multiple generations. One intriguing possibility is that different bacterial endosymbionts may help provision planthoppers with essential amino acids, much like they do in aphids. Recently, Tang et al. (2010) identified 18 distinct bacterial operational taxonomic units (OTUs) from *N. lugens*, and found that some OTUs were present or absent in different planthopper populations specialized on TN1, Mudgo, and ASD7. At this point, it is unclear which bacteria are obligate and which are facultative endosymbionts, and what role they may play in host nutrition. A possible corollary to the finding by Tang et al. (2010) is that planthopper specialization on different rice varieties could be a function in a shift on the planthopper bacterial endosymbiont community. This could help explain why aposymbiotic planthoppers also showed an increase in the proportional concentrations of rarer amino acids. Fundamental questions emerge regarding the role of these bacterial endosymbionts in planthopper amino acid metabolism. Could bacterial endosymbionts be responsive to plant variation? In aphids, the abundance of facultative secondary endosymbionts is thought to be mediated by their ability to provide nutrients, such as supplementing the supply of amino acids (Gil et al., 2002). Wilkinson et al. (2001) found that the density of secondary symbiotic bacteria increased in aphids on a high sucrose:amino acid diet, and that plants may mediate the disruption of symbiotic bacterial populations. However, bacterial symbiont composition is not thought to be determined by the nutritional content of their host plants (Douglas et al., 2006). Additional study would help determine how rice varieties that vary nutritionally and defensively may interact with yeast-like endosymbionts and bacterial symbionts in *N. lugens*.

We found that planthoppers reared on different rice varieties demonstrated a trade-off in growth, when exposed to non-natal varieties. However, we did not detect that the rice variety contrib-

uted to any trade-off in planthopper amino acid composition. Extended planthopper rearing decreased non-limiting amino acids, and increased the abundance of rarer amino acids. The physiological “adaptation” of *N. lugens* to rice varieties could be linked to a change in protein expression, or amino acid metabolism. Given that this process appears to be inevitable when planthoppers are repeatedly reared on resistant rice varieties, understanding how planthoppers physiologically “adapt” to resistant varieties will help inform the development of resistant rice varieties for this economically important and cosmopolitan pest.

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Appendix A

The AccQ-Fluor amino acid derivatives were separated on a high performance liquid chromatography system. The following gradient below was used in the chromatography. Eluent A was Waters AccQ Tag Eluent A Concentrate (WAT052890) and Eluent B was 60% acetonitrile.

Time	Flow	%A	%B	Curve
0	1	100	0	6
0.5	1	98	2	6
15	1	95	5	6
19	1	90	10	6
32	1	67	33	6
33	1	67	33	6
35	1	0	100	6
39	1	0	100	6

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