Gut Microbes Contribute to Nitrogen Provisioning in a Wood-Feeding Cerambycid

PAUL AYAYEE,[1,2] CRISTINA ROSA,[3] JAMES GREGORY FERRY,[4] GARY FELTON,[2] MIKE SAUNDERS,[2] AND KELLI HOOVER[2,5]

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ABSTRACT Xylophagous insects often thrive on nutritionally suboptimal diets through symbiotic associations with microbes that supplement their nutritional requirements, particularly nitrogen. The wood-feeding cerambycid *Anoplophora glabripennis* (Motschulsky) feeds on living, healthy host trees and harbors a diverse gut microbial community. We investigated gut microbial contributions to larval nitrogen requirements through nitrogen fixing and recycling (urea hydrolysis) processes, using a combination of molecular, biochemical, and stable isotope approaches. Genes and transcripts of conserved regions of the urease operon (*ureC*) and nitrogen fixing (*nif*) regulon (*nifH*) were detected in *A. glabripennis* eggs and larvae from naturally infested logs and from larvae reared on artificial diet. Significant nitrogen fixation and recycling were documented in larvae using $^{15}N_2$ gas and ^{15}N-urea, respectively. Subsequent ^{15}N-routing of incorporated recycled nitrogen into larval essential and nonessential amino acids was shown for ^{15}N-urea diet-fed larvae. Results from this study show significant gut microbial contributions to this insect's metabolic nitrogen utilization through nitrogenous waste product recycling and nitrogen fixation.

KEY WORDS amino acid, xylophagy, stable isotope, nitrogen fixation, urea hydrolysis

Nitrogen is a limiting element for terrestrial insect herbivores in general and wood-boring insects in particular (Mattson 1980) due to its importance in protein and nucleotide biosynthesis and the production of other essential metabolites necessary for growth and development (Sterner and Elser 2002). Wood-feeding insects live on nutritionally unbalanced food sources (wood) with low nitrogen content (0.03–0.1% nitrogen; Mattson 1980). The majority of the available nitrogen in wood is mainly in the form of intractable cell wall structural glycoproteins, with some in the form of alkaloids and only ≈0.001% available as free amino acids (Watkinson et al. 2006). Nitrogen limitation is therefore a particularly extreme nutritional obstacle facing wood-feeding insects. Several wood-feeding insects are proposed to harbor symbiotic gut microbes that could contribute to overcoming nitrogen limitation via nitrogen fixation (Nardi et al. 2002) and nitrogenous waste recycling (Dillon and Dillon 2004).

The Asian longhorned beetle, *Anoplophora glabripennis* (Motschulsky) (Coleoptera: Cerambycidae: Lamiini), a wood-feeding insect native to China and Korea (Haack et al. 1997), is known to harbor a diverse assemblage of gut microbes, with microbial functions speculated to be beneficial to the insect host (Schloss et al. 2006, Geib et al. 2009). It is polyphagous in its native ranges (Nowak et al. 2001) as well as in its introduced range in North America, with 47 different host tree species recorded worldwide (Yang et al. 1995, Haack et al. 2010). It is unusual for a cerambycid that feeds on living healthy trees to have such a broad host range (Hanks 1999). Eggs deposited by adult females usually hatch in ≈2 wk, with early instar larvae initially feeding in the cambial layer of host trees, while older larvae move deeper into the tree feeding on sapwood and heartwood (Haack et al. 2010). Complete developmental time in the wood ranges from 1 to 2 yr depending on environmental temperatures (Haack et al. 2010), and larvae are expected to be markedly impacted by nitrogen limitation in their woody substrate. The gut microbial community of *A. glabripennis* is proposed to supplement larval nitrogen utilization through nitrogenous waste recycling via urea hydrolysis, nitrogen fixation, or both.

Information about nitrogen recycling of insect nitrogenous waste products (urea) by gut microbes associated with wood-feeding insects feeding on healthy host trees (not predigested by wood decay fungi) is scant. Ureases, the enzymes responsible for urea hydrolysis, are present in some free-living as well as symbiotic bacteria, fungi, and plants, and are important components of both plant and microbial nitrogen

[1] Department of Evolution, Ecology and Organismal Biology, the Ohio State University, Columbus, OH 43210.
[2] Department of Entomology and Center for Chemical Ecology, The Pennsylvania State University, University Park, PA 16802.
[3] Department of Plant Pathology and Environmental Microbiology, the Pennsylvania State University, University Park, PA 16802.
[4] Department of Biochemistry, Microbiology and Molecular Biology, Pennsylvania State University, University Park, PA 16802.
[5] Corresponding author, e-mail: kxh25@psu.edu.

metabolism processes. Urea ($(NH_2)_2CO$) is hydrolyzed to ammonia (NH_3) and carbon dioxide (CO_2) under the enzymatic action of the enzyme urease as follows: $(NH_2)_2CO + H_2O \rightarrow^{\text{(urease dependent)}} \rightarrow NH_3 + H_2NCOOH \rightarrow^{\text{(urease independent)}} \rightarrow CO_2 + 2NH_3$ (Mobley and Hausinger 1989).

Alternatively, nitrogen fixation from the atmosphere by bacteria and Archaea is also posited to contribute significantly to the total nitrogen budget of terrestrial wood-feeding insects such as *A. glabripennis* (Nardi et al. 2002, Dillon and Dillon 2004). Biological nitrogen fixation is an entirely microbial process accounting for ≈140 million tons of nitrogen input in terrestrial systems (Nardi et al. 2002). Nitrogen fixation is catalyzed by the microbial nitrogenase enzyme, which is encoded by the *nif* regulon consisting of several operons with genes that are transcribed and translated to form the nitrogenase enzyme and the regulatory proteins involved in nitrogen fixation (Zehr et al. 2003), which are lacking in insects. The overall biochemical reaction for nitrogen fixation is represented as: $N_2 + 8H^+ + 16ATP + 8e^- \rightarrow 2NH_3 + H_2 + 16ADP + 16Pi$.

The ammonia (NH_3) generated from both urea hydrolysis and nitrogen fixation is first incorporated into the organism (plant or microbe) via the glutamine synthetase (GS) and the glutamine 2-oxoglutarate amidotransferase (GOGAT) enzyme complex (GS–GOGAT), and used subsequently in the biosynthesis of other amino acids (Mobley and Hausinger 1989). This enzyme complex represents a common link between the two nitrogen metabolism pathways of nitrogen fixation and nitrogen recycling (Merrick and Edwards 1995), and this coupling is postulated to ensure the efficient utilization of nitrogen under nitrogen-limiting conditions (Hongoh et al. 2008).

In this study, we used culture-independent approaches (PCR and reverse transcription PCR [RT-PCR]) to verify the presence of urea-hydrolyzing and nitrogen-fixing members of the gut microbial community of *A. glabripennis* larvae from naturally infested host trees and from larvae fed on artificial diet. We also investigated the presence of urea-hydrolyzing and nitrogen-fixing members in *A. glabripennis* eggs. This was carried out through the detection of the conserved *ureC* genes and transcripts of the urease operon as well as the conserved *nifH* gene and transcripts of the *nif* regulon of urea-hydrolyzing and nitrogen-fixing bacteria, respectively. Bulk (whole tissue) and compound-specific (amino acid) isotope analysis were respectively used to quantify the incorporation of recycled ^{15}N from microbial ^{15}N-urea hydrolysis into larval biomass ($\delta^{15}N$), and specific routing of recycled incorporated ^{15}N into larval amino acids ($\delta^{15}N_{AA}$). Nitrogen fixation was indirectly investigated in *A. glabripennis* larvae via the acetylene reduction assay, and directly using $^{15}N_2$ gas enrichment and supplementation experiments, followed by stable isotope ratio mass spectrometry to verify and quantify fixed and incorporated ^{15}N in larval biomass.

Materials and Methods

Sources of Insects. *A. glabripennis* larvae used for DNA and RNA extractions were obtained from naturally infested red maple logs (*Acer rubrum* L.) from Worcester and Bethel, OH, and from sugar maple logs (*Acer saccharum* L.) in which females reared on artificial diet (Keena 2005) had oviposited (referred to as oviposition logs). Briefly, the artificial diet was sterilized by boiling before adding vitamins and contains the antimicrobial components sorbic acid, methyl paraben, and sodium propionate. Carbon and protein sources in the diet include wheat germ, casein, cellulose, and Torula yeast. Eggs used for DNA and RNA extraction were obtained from sugar maple oviposition logs from colony-reared beetles (original source from a mixed population from Worcester, Chicago, China, and New York). Oviposition of viable eggs by this insect requires mated beetles that have reached sexual maturation, after feeding on host trees for ≈2 wk. Larvae used for ^{15}N-labeled urea artificial diet experiments were obtained from Norway maple trees (*Acer plantanoides* L.) reared in the quarantine greenhouse facility at Penn State University (parents were from the PSU colony). Larvae used for acetylene reduction assays were estimated by head capsule width to be between late second and early fourth instars and were collected from naturally infested red maple logs. Larvae used for $^{15}N_2$ gas experiments were between early second to late third instars and were obtained from Norway maple trees reared in the quarantine greenhouse facility at Penn State University (parents were from the PSU colony). DNA and RNA for positive controls were extracted from whole worker termites of *Reticulitermes flavipes* Kollar, a species known to harbor urease-producing microbes (Bentley 1984). Termites were obtained from rotting wood at a local neighborhood park in the Park Forest neighborhood in State College, PA. No permit was obtained for this purpose. Transportation of logs from U.S. infestations and maintenance of the quarantine insect colony were under U.S. Department of Agriculture–Animal and Plant Health Inspection Service Permit No. P526P-11-02877.

DNA and RNA Extraction From *A. glabripennis* Developmental Stages. Larvae and eggs were surface sterilized by rinsing them once in 10% CoveragePlus (Steris, Mentor, OH) followed by two rinses in DNAase-free water. Gut samples were placed in MT lysis buffer solution (MP Biomedicals, Solon, OH). Depending on the size of the insects, guts were processed in pools or separately, while eggs were pooled in groups of three to obtain sufficient DNA. After homogenization using the FastPrep Instrument (BIO101 Inc, LA Jolla, CA) at 6 m/s for 45 s, total DNA was extracted from samples (FastDNA SPIN for Soil Kit; MP Biomedicals, Solon, OH) according to the manufacturer's protocol. Extracted DNA was quantified using the NanoDrop 2000 UV-VIS spectrophotometer (Thermo Scientific, Wilmington, DE) and stored at −20°C until use.

For RNA extraction, eggs and larvae were surface sterilized as described above and RNA was extracted using PowerSoil RNA (Mo Bio, Carlsbad, CA) according to the manufacture's protocol, after homogenization with a Vortex Genie-2 (Scientific industries, Bohemia, NY) at 3,200 rpm for 15 min. RNA was quantified as described above and stored at −80°C until needed.

Detection of Microbial *ureC* and *nifH* Genes From DNA Extracted From Eggs and Larval Guts. The eubacteria primer pair E8F (5′-GAGTTTGATCCTG GCTCA-3′) and E1541R (5′-AAGGAGGTGATC CANCCR-3′; Baker et al. 2003) was used to verify the presence of microbial DNA and to amplify the V1 to V8 variable regions of 16S rRNA gene (expected PCR product 1533 bp) from extracted DNA. The PCR master mix consisted of 12.5 μl of Promega Gotaq Green Master mix (1× final concentration), 2.5 μM of appropriate forward and reverse primers, and 80 ng of template DNA. The PCR conditions for 16S rRNA were as follows: 95°C for 4 min, 35 cycles at 94°C for 40 s, 56.5°C for 2 min, and 72°C for 2 min, with a final extension at 72°C for 7 min. 16s rRNA PCR products were analyzed by electrophoresis on a 1.2% agarose gel using a 1 kb molecular weight ladder (Promega Corporation, Madison, WI).

The degenerate universal L2F/L2R *ureC* primer pair (Gresham et al. 2007) was used to amplify a 388 bp fragment of the *ureC* gene. The PCR master mix was the same as described above with final concentrations of 2.5 μM of the appropriate forward and reverse primers, and 80 ng of template DNA. PCR conditions were as follows: 97°C for 5 min, 40 cycles at 96°C for 1:50 min, 54.2°C for 2 min, and 72°C for 2 min, with a final extension at 72°C for 10 min. DNA extracted from termite workers served as the positive controls and nuclease-free water (Promega Corporation) as the negative control. Microbial *ureC* PCR products were analyzed on a 1.8% agarose gel and compared with a 1 kb molecular weight ladder (Promega Corporation).

The *nifH* primers were used to amplify a 450 bp fragment of the *nifH* gene (Pinto-Tomas et al. 2009). The PCR master mix was the same as described above with final concentrations of 2.5 μM of the appropriate forward and reverse primers, and 80 ng of template DNA. DNA extracted from termite workers served as the positive control and nuclease-free water (Promega Corporation) as the negative control. The PCR conditions for *nifH* were as follows: 95°C for 5 min, 40 cycles of 94°C for 11 s, 92°C for 15 s, 54°C for 8 s, 56°C for 30 s, 74°C for 10 s, and 72°C for 10 s, and final extension for 10 min at 72°C. Microbial *nifH* PCR products were analyzed on a 1.8% agarose gel using a 1 kb molecular weight ladder (Promega Corporation) for size comparison.

Detection of Microbial *ureC* and *nifH* Transcripts From RNA Extracted From Eggs and Larval Guts. RT-PCR was performed using both the degenerate universal *ureC* primer pair L2F/L2R (Gresham et al. 2007) and *nifH* primers. Absence of DNA contamination of the RNA was confirmed by PCR on selected RNA samples. RNA extracted from termite workers served as the positive control for *ureC* transcripts and nuclease-free water as the negative control. One-step RT-PCR was performed on extracted RNA. The RT-PCR reaction master mix was prepared as described above with the addition of 7 U of SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA) per reaction to the master mix followed by addition of the appropriate primers. Approximately 90 ng of template RNA was added to each RT-PCR reaction. cDNA synthesis was conducted at 42°C for 60 min with an inactivation step at 70°C for 10 min, followed by the respective *ureC* and *nifH* PCR conditions as described for DNA samples in section Detection of Microbial *ureC* and *nifH* Genes From DNA Extracted From Eggs and Larval Guts.

Quantification of Incorporated Bulk Recycled Nitrogen Into *A. glabripennis* Larval Biomass Using Isotope Ratio Mass Spectrometry. To verify that recycled nitrogen from bacterial urease activity is incorporated into insect biomass, we added ^{15}N-labeled urea (hereafter ^{15}N-urea) to the artificial diet specific for this species (Keena 2005). To obtain larvae for this experiment, four *A. glabripennis* male and female adults were released into cages containing two Norway maple trees for ~3 wk to allow time for adequate oviposition. After 3 mo, developing larvae were removed from trees and individuals were randomly assigned to one of the three groups as follows: larvae fed on unaltered artificial diet (artificial diet-fed larvae), larvae fed on artificial diet plus 2.7 mM ^{14}N-urea (^{14}N-urea diet-fed larvae), and larvae fed on artificial diet plus 2.7 mM ^{15}N-urea (^{15}N-urea diet-fed larvae). Artificial diets were made as described previously (Keena 2005) but modified by the omission of antimicrobial components. All larvae were reared for 2 wk on their respective diets, and diets were changed after 1 wk to minimize potential microbial contamination. After 2 wk, larvae were surface sterilized in 10% CoveragePlus (Steris), whole guts removed and discarded, and larval cadavers freeze-dried individually for 48 h. The entire gut was removed to prevent confounding of larval ^{15}N incorporation data with microbial ^{15}N incorporation.

Each dried larval cadaver was ground using a ball mill grinder, weighed, wrapped in tin foil, and placed in the autosampler of a Finnegan MAT Delta E Isotopic Ratio Mass Spectrometer (IRMS) coupled to a gas chromatograph (GC) via a combustion interface (C). The IRMS quantifies the atom percent abundance of ^{14}N and ^{15}N in each sample compared with known gaseous and nongaseous standards. The ^{14}N and ^{15}N atom percent abundance values were then used to determine the bulk δ^{15}N (^{15}N/^{14}N) values of larval samples and expressed as $[(R_{smpl}/R_{stnd}) - 1] \times 1{,}000‰$, where R is the ratio of heavy to light isotope in the sample, which is designated as R_{smpl}, and R_{stnd} is the standard (Scrimgeour and Robinson 2008). IRMS analyses were performed in the laboratory of Dr. Alexander Hristov at Penn State University, University Park.

Quantification of Recycled Nitrogen Routing Into Amino Acids Using Compound-Specific Isotope Analysis. Aliquots of the gut samples from the two treatment groups from above (^{14}N-urea diet-fed larvae and

^{15}N-urea diet-fed larvae; $n = 3$ each) and a freeze-dried and ground sample of the unaltered artificial diet itself ($n = 1$) were sent to the stable isotope facility at University of California, Davis for amino acid analysis.

Briefly, samples were acid hydrolyzed (http://stableisotopefacility.ucdavis.edu-/gcaminosample) and derivatized in a solution of methanol, pyridine, and methyl chloroformate using a one-step rapid derivatization method (Chen et al. 2010). Compound-specific isotope-amino acid analysis was used to quantify $^{15}N_{AA}/^{14}N_{AA}$ isotope values of essential and nonessential amino acids in the three treatment groups ($\delta\ ^{15}N_{AA}$); all samples were run in duplicate.

Indirect Measurement of Nitrogen Fixation Using the Acetylene Reduction Assay. For the acetylene reduction assay, six larvae between late second and early fourth instars were surface sterilized and placed in 20 ml vials with moistened swabs. The vials were sealed and 4 ml of air was removed and replaced with 4 ml of acetylene to create a 20% (vol:vol) acetylene and air mixture. The negative control consisted of two gypsy moth larvae, which does not fix nitrogen, placed in a separate vial with 20% (vol:vol) acetylene and air mixture. An empty vial with only 20% (vol:vol) acetylene and air and another vial with 45 worker termites in a 20% (vol:vol) acetylene and air mixture served as the negative and positive controls, respectively. The experiment was repeated twice and in each case, the weight of the experimental insects were measured and used to calculate the acetylene reduction rate as the production of mmole ethylene/g/h for each treatment.

The vials were incubated for 24 h in darkness at 28°C, and the reduction of acetylene to ethylene was measured by sampling 0.1 ml of the headspace from each vial at 15-min intervals for 1 h. Ethylene measurements were carried out with a Hewlett–Packard GC (HP-5890 GC, Palo Alto, CA) equipped with a single flame ionization detector, an 80 or 100 Porapak Q column (6 feet × 1/8 inch), with helium gas at 40 ml/min as the carrier, and an injector temperature of 70°C.

Direct Quantification of Nitrogen Fixation Using $^{15}N_2$ Gas Enrichment. We investigated insect incorporation of fixed nitrogen using the heavy nitrogen isotope ^{15}N. To obtain larvae for this experiment, four *A. glabripennis* male and female adults were released into cages containing two Norway maple trees for ~3 wk. After 3 mo, the trees were cut into appropriate-sized bolts, and bolts were surface sterilized with a 10% bleach solution. Bolt ends were coated with a sealant (Anchor-seal Inc., Gloucester, MA) to minimize desiccation and placed into airtight 3-liter glass jars containing a carbon dioxide trap (soda lime in cell culture tubes) with inlet and outlet valves.

The $^{15}N_2$ gas was generated according to (Diocares et al. 2006) $(^{15}NH_4)_2SO_4 + 2LiOBr \rightarrow Li_2SO4 + {}^{15}N_{2\ (g)} + 2BrO^- + 4H_{2\ (g)}$; the generated nitrogen gas was verified at the Cornell University Stable Isotope laboratory (Ithaca, NY) to be made up of 100% ^{15}N isotopes. Treatment jars were flushed first and filled with pure oxygen, and $^{15}N_2$ gas was injected into treatment jars. The amount of $^{15}N_2$ gas injected was calculated as follows: 1 U of $(^{15}NH_4)_2SO_4$ (1.8 mmol) produces one unit of $^{15}N_2$ gas (1.8 mmol) based on the equation above. The $(^{15}NH_4)_2SO_4$ used was 98% ^{15}N. The amount of ^{15}N isotopes in the generated $^{15}N_2$ gas was therefore: 0.98 by 1.8 mmol = 1.764 mmol per reaction. Six such reactions were performed and the generated gas injected into the treatment jars. The total amount of ^{15}N in each treatment jar was therefore: 1.764 mmol × 6 = 10.584 mmol or 0.010584 mol. This is the mole fraction of ^{15}N in each treatment jars, and the mole fraction is the same as the volume fraction at standard temperature and pressure (http://acmg.seas.harvard.edu/people/faculty/djj/book/bookchap1.html). Using the formula, 1 ppmv = mole fraction × 1,000,000, the concentration of ^{15}N in each treatment jar in ppmv was, therefore, 0.010584 by 1,000,000 = 10,584 ppmv. Control jars were flushed with pure oxygen and filled with an 80% N_2: 20% O_2 gas mixture. The natural amount of ^{15}N in the control jars was determined as follows: the amount of N_2 in atmospheric air is 780,840 ppmv. Of this amount, 0.36% is ^{15}N (Junk and Svec 1958); we calculated the amount of ^{15}N in atmospheric air as follows: 0.0036 by 780,840 ppmv = 2,811.024 ppmv. Thus, the amount of ^{14}N in each control jar was 778,028.976 ppmv.

Statistical Analyses. To determine whether a significant amount of recycled nitrogen was incorporated into larval biomass, bulk δ^{15}N data were analyzed using a one-way analysis of variance (ANOVA) with experimental group as model effects (independent variable) and $\delta\ ^{15}$N as the dependent variable. To determine whether the recycled ^{15}N from urease hydrolysis incorporated into larval biomass was used to synthesize essential, nonessential, or both, amino acids, a multivariate nested ANOVA (Larsen et al. 2009) was performed using amino acid identity, treatment group, and their interaction as fixed effects and raw amino acid $\delta\ ^{15}$N values as the dependent variable. Analyses were done separately for essential and nonessential amino acids.

Acetylene reduction results were analyzed using a one-way ANOVA with experimental groups as model effects (independent effects) and acetylene reduction rates as the dependent variables. Bulk δ^{15}N results from the ^{15}N nitrogen fixing experiment were analyzed using a one-way ANOVA with experimental groups as model effects (independent variables) and $\delta\ ^{15}$N as the dependent variable. All statistical analyses and means separation by Tukey's HSD were performed using JMP 10 (SAS Inc, NC).

Results

Detection of Microbial *ureC* Genes and Transcripts From DNA and RNA Extracted From Eggs and Larval Guts. In total, 16 DNA samples extracted from larval guts and eggs were positive for microbial 16S rRNA genes (Table 1). The microbial *ureC* PCR gene was detected in 11 of these 16 samples (68.7%) using the L2F/L2R *ureC* primer pair (Table 1). Microbial *ureC* transcripts were detected in 16 of 19 larval gut and egg

Table 1. PCR and RT-PCR detection of microbial 16S rRNA and microbial *ureC* genes and transcripts, respectively, from DNA and RNA samples extracted from eggs and the guts of larval *A. glabripennis*, using the universal degenerate *ureC* primer pair L2F/L2R

Source	PCR results		
	Insect stage	16S rRNA	*ureC*
ureC genes			
Sugar maple	Eggs ($n = 2$, pools of 3 eggs)	2/2	2/2
Maple logs	First and second instars ($n = 2$)	2/2	1/2
Maple logs	Fourth and fifth instars ($n = 2$)	2/2	2/2
Artificial diet	Second and third instars ($n = 4$)	4/4	2/4
Artificial diet	Fourth and fifth instar ($n = 6$)	6/6	4/6
	RT-PCR results		
	Insect stage		*ureC*
ureC transcripts			
Sugar maple	Eggs ($n = 2$, pools of 3 eggs)		1/2
Maple logs	First and second instars ($n = 2$)		2/2
Maple logs	Fourth and fifth instar ($n = 3$)		3/3
Artificial diet	First and second instars ($n = 4$)		4/4
Artificial diet	Third and fourth instars ($n = 4$)		2/4
Artificial diet	Fifth instars ($n = 4$)		4/4

Numbers in brackets are the number of replicates of DNA and RNA samples of each developmental stage; number of samples positive for genes of interest shown in the numerators, while numbers of replicates appear in the denominators.

RNA samples (84.2%) using the same primer pair (Table 1).

Detection of Microbial *nifH* Genes and Transcripts From DNA and RNA Extracted from Eggs and Larval Guts. In total, 18 DNA samples from eggs and the gut of larvae reared on artificial diet and larvae from naturally infested logs were positive for microbial 16S rRNA genes. Microbial *nifH* genes were detected in 16 of these 18 DNA samples including eggs as well as both larvae reared on artificial diet and from naturally infested logs (Table 2). Similarly, microbial *nifH* transcripts were also detected in 16 of the 17 RNA samples, including eggs, as well as first- to fifth-instar larvae reared on artificial diet, and second- to fourth-instar larvae collected from naturally infested logs (Table 2).

Table 2. PCR and RT-PCR detection of microbial 16S rRNA and microbial *nifH* genes and transcripts, respectively, from DNA and RNA samples extracted from eggs and the guts of larval *A. glabripennis*

Source	PCR results		
	Insect stage	16S rRNA	*nifH*
Maple logs	Eggs ($n = 2$ pools of 3 eggs)	2/2	2/2
Maple logs	First to second instars ($n = 2$)	2/2	2/2
Maple logs	Third to fourth instars ($n = 4$ pools of 5 larvae)	4/4	4/4
Artificial diet	Second to third instars ($n = 4$)	4/4	4/4
Artificial diet	Fourth to fifth instars ($n = 6$)	6/6	4/6
	RT-PCR results		
	Insect stage		*nifH*
Maple logs	Eggs ($n = 2$ pools of 3 eggs)		2/2
Maple logs	Second to fourth instars ($n = 3$)		3/3
Artificial diet	First to third instars ($n = 6$)		6/6
Artificial diet	Fourth to fifth instars ($n = 6$)		5/6

Numbers in brackets are the number of replicates of DNA and RNA samples of each developmental stage; number of samples positive for genes of interest shown in the numerators, while numbers of replicates appear in the denominators.

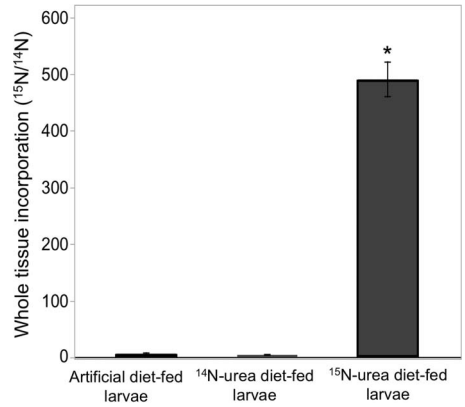

Fig. 1. Bulk ^{15}N ($\delta\,^{15}N$) incorporated into the biomass of artificial diet-fed larvae, ^{14}N-urea diet-fed larvae, and ^{15}N-urea diet-fed larvae, measured via isotope ratio mass spectrometry. One-way ANOVA: $F_{(2,11)} = 244$; $P < 0.0001$. Shown are mean values for four replicates per treatment; error bars represent the standard error of the mean. * Represents statistical significance at $P < 0.05$.

Quantification of Incorporated Recycled Nitrogen Into Larval Biomass and Amino Acids Using Isotope Ratio Mass Spectrometry. There was significant incorporation of recycled nitrogen into larval biomass. Mean $\delta\,^{15}N$ values of ^{15}N-urea diet-fed larvae was 70-fold and 100-fold greater than the $\delta\,^{15}N$ values of artificial diet-fed larvae and ^{14}N-urea diet-fed larvae, respectively (Fig. 1). Raw $\delta\,^{15}N$ data used for analysis from IRMS are presented in Table 3.

We obtained consistent analytical separation for 10 amino acids following corrections of $\delta\,^{15}N$ isotope values relative to corresponding amino acid standards.

Table 3. Raw $\delta^{15}N$ ($^{15}N/^{14}N$) values for larvae removed from the artificial diets groups used in the ^{15}N-urea hydrolysis experiment

Experimental group	Sample ID	Instar	$\delta^{15}N$ (‰)
Normal diet-fed larvae	CT1–13–1	Late second	12.16
Normal diet-fed larvae	CT1-13–2	Early third	9.53
Normal diet-fed larvae	CT1-18–1	Early third	7.52
Normal diet-fed larvae	CT1-15–1	Early third	6.66
Normal diet-fed larvae	CT1-15–2	Early third	5.03
Normal diet-fed larvae	CT1-17–1	Early third	4.17
Normal diet-fed larvae	CT1-17–2	Early third	3.94
^{14}N-urea diet-fed larvae	CT2-4–1	Late third	5.17
^{14}N-urea diet-fed larvae	CT2-9–1	Early third	4.91
^{14}N-urea diet-fed larvae	CT2-9–2	Early third	4.61
^{14}N-urea diet-fed larvae	CT2-10–1	Early third	4.77
^{14}N-urea diet-fed larvae	CT2-10–2	Early third	4.81
^{14}N-urea diet-fed larvae	CT2-3–1	Early third	4.62
^{14}N-urea diet-fed larvae	CT2-3–2	Early third	4.51
^{15}N-urea diet-fed larvae	T-2-1	Early third	541.04
^{15}N-urea diet-fed larvae	T-2-2	Early third	551.41
^{15}N-urea diet-fed larvae	T-6-1	Early third	436.62
^{15}N-urea diet-fed larvae	T-6-2	Early third	429.79
^{15}N-urea diet-fed larvae	T-14-1	Early third	440.99
^{15}N-urea diet-fed larvae	T-14-2	Early third	441.37
^{15}N-urea diet-fed larvae	T-12-1	Late third	542.50

Table 4. Individual amino acid $^{15}N_{AA}/^{14}N_{AA}$ values ($\delta^{15}N_{AA}$) from larvae and the artificial diet obtained via compound-specific isotope ratio mass spectrometry analysis of amino acids

Groups	Ile	Leu	Lys	Phe	Val	Ala	Asp	Glu	Gly	Pro
AD	−0.42	1.82	−1.3	2.75	2.35	1.44	−0.24	0.6	3.03	−0.39
^{15}N-urea diet-fed larvae 1	158.7	60.8	−5.6	35.43	100.6	727.97	441.7	689.3	329.6	75.7
^{15}N-urea diet-fed larvae 2	105.2	41.3	−3.6	23.8	101.4	501.9	285.7	460.4	225.0	58.5
^{15}N-urea diet-fed larvae 3	166.4	64.1	−2.8	30.39	115.0	759.4	415.4	593.6	342.8	122.6
Normal diet-fed larvae 1	6.27	7.67	−0.1	6.75	6.1	4.17	1.57	6.19	3.4	7.24
Normal diet-fed larvae 2	5.96	7.03	0.43	4.56	4.03	4.26	18.34	7.44	3.4	8.19
Normal diet-fed larvae 3	0.81	4.19	1.06	4.46	3.79	2.37	1.05	4.84	−0.1	6.39

The essential amino acids (EAA) quantified were leucine (Leu), isoleucine (Ile), lysine (Lys), phenylalanine (Phe), and valine (Val). Nonessential amino acids (NEAA) were alanine (Ala), aspartate (Asp), glutamate (Glu), glycine (Gly), and proline (Pro). Individual amino acid δ^{15}N values from the larval and artificial diet groups are presented in Table 4.

There was significant routing of recycled nitrogen into both EAAs and NEAAs (Fig. 2). Total δ^{15}N routed into EAA ($\delta^{15}N_{EAA}$) of ^{15}N-urea diet-fed larvae was 16- and 66-fold greater than the $\delta^{15}N_{EAA}$ of ^{14}N-urea diet-fed larvae and the artificial diet, respectively ($F_{(2,32)} = 122$; $P < 0.0001$; Fig. 2). Similarly, total δ^{15}N routed into NEAA ($\delta^{15}N_{NEAA}$) of ^{15}N-urea diet-fed larvae was 77- and 446-fold greater than the $\delta^{15}N_{NEAA}$ of ^{14}N-urea diet-fed larvae and the artificial diet, respectively ($F_{(2,32)} = 150$; $P < 0.0001$; Fig. 2). Overall, within the ^{15}N-urea diet-fed larval group, there was lower ^{15}N-routing into EAA $\delta^{15}N_{EAA}$ (66 ‰ ± 3.30; mean ± SEM) relative to ^{15}N-routing into NEAA, $\delta^{15}N_{NEAA}$ (402 ‰ ± 17.4), with ≈83.5% of the recycled nitrogen routed into nonessential amino acids.

Delta ^{15}N ($\Delta^{15}N = \delta^{15}N_{Larvae} - \delta^{15}N_{Artificial\ diet}$; Larsen et al. 2009) values were calculated for the EAA and NEAA of larval samples to determine δ^{15}N isotopic enrichment or depletion relative to the artificial diet amino acids (Fig. 3a and b). Analyses showed a significant diet and essential amino acid δ^{15}N-enrichment interaction for the ^{15}N-urea diet-fed larvae relative to the other treatment groups (Group × amino acids; $F_{(8,26)} = 21$; $P < 0.0001$; Fig. 3b). Results showed differences in the relative enrichments of ^{15}N into the essential amino acids of ^{15}N-urea diet-fed larvae, with Ile, Leu, and Val being the most enriched, and Phe and Lys being the least ^{15}N-enriched, relative to the same amino acids from the control groups. There were no significant differences in ^{15}N-enrichment between the ^{14}N-urea diet-fed larvae and the artificial diet itself (Fig. 3b).

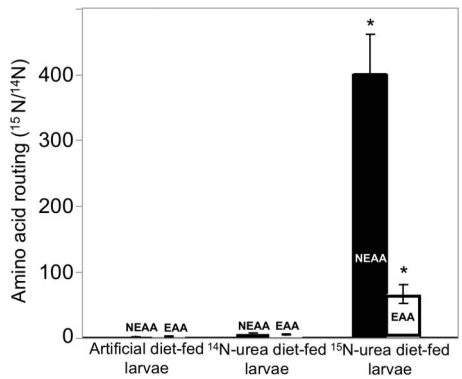

Fig. 2. The amount of ^{15}N (δ^{15}N) routed into EAA and NEAA of artificial diet-fed larvae, ^{14}N-urea diet-fed larvae, and ^{15}N-urea diet-fed larvae. Essential and nonessential amino acids ANOVA: $F_{(2,32)} = 122$; $P < 0.0001$, and $F_{(2,32)} = 150$; $P < 0.0001$, respectively. Shown are mean values for three replicates per larval group and one for the artificial diet control. Error bars represent standard error of the mean. * Represents statistical significance at $P < 0.05$.

Analyses also showed a significant diet and nonessential amino acid interaction for the ^{15}N-urea diet-fed larvae relative to the other treatment groups ($F_{(8,26)} = 10$; $P < 0.0001$; Fig. 3a). Similarly, not all nonessential amino acids were equally ^{15}N-enriched. Ala was the most enriched, followed by Asp, Glu, and Gly, which all differed significantly from the same amino acids in the control groups. Proline was the least enriched and not significantly different from the controls. There were no significant differences within or between the $\delta^{15}N_{NEAA}$ values from the two control groups (Fig. 3a).

Indirect and Direct Measures of Nitrogen Fixation in Larvae. Acetylene reduction by *A. glabripennis* larvae (0.58 ± 0.23 mmol ethylene/g/h; mean ± SEM) was significantly higher compared with the gypsy moth negative control (−0.33 ± 0.23 mmol ethylene/g/h; Fig. 4), indicative of the presence of a nitrogenase enzyme complex in larvae. The rate for *A. glabripennis* was, however, significantly lower than for termites (5.17 ± 0.23 mmol ethylene/g/h).

The bulk amount of ^{15}N incorporated into larvae exposed to $^{15}N_2$ gas was 1.6 fold higher than that of control larvae ($F_{(1,7)} = 12$; $P > 0.014$; Fig. 5), representing an increase in ^{15}N of ≈36% in the ^{15}N-enriched gas larvae. Raw δ^{15}N data used for analysis from IRMS are presented in Table 5.

Discussion

Microbial assistance is required for nitrogen provisioning in some insects because insects generally excrete their nitrogenous waste products (uric acid or urea) without the ability to recycle them (Mobley and Hausinger 1989) nor can insects fix atmospheric nitrogen on their own (Douglas 2009). Microbial nitrogen provisioning in wood-feeding insect hosts can

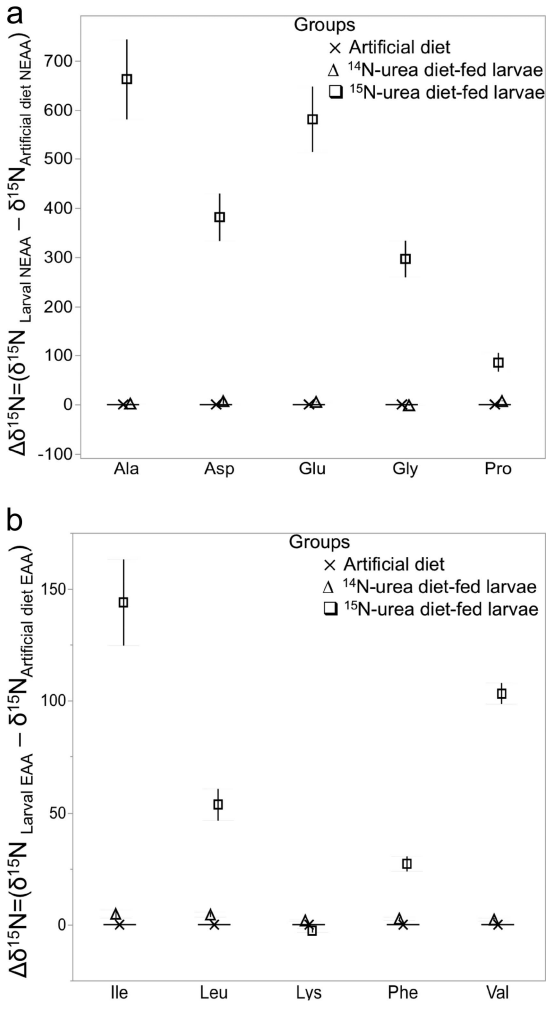

Fig. 3. (a) $\delta^{15}N$ isotope enrichment or depletion in the nonessential amino acids ($\delta^{15}N_{NEAA}$) of larval *A. glabripennis* treatment groups relative to the nonessential amino acids in artificial diet ($\Delta\delta^{15}N = \delta^{15}N_{Larvae\ NEAA} - \delta^{15}N_{Artificial\ diet\ NEAA}$). (b) $\delta^{15}N$ isotope enrichment or depletion in the essential amino acids ($\delta^{15}N_{EAA}$) of larval treatment groups relative to the essential amino acids in artificial diet ($\Delta\delta^{15}N = \delta^{15}N_{Larvae\ EAA} - \delta^{15}N_{Artificial\ diet\ EAA}$). Shown are mean values for three replicates per group; error bars represent standard error of the mean. Nonessential amino acids were Ala, Asp, Glu, Gly, and Pro. Essential amino acids were Leu, Ile, Lys, Phe, and Val.

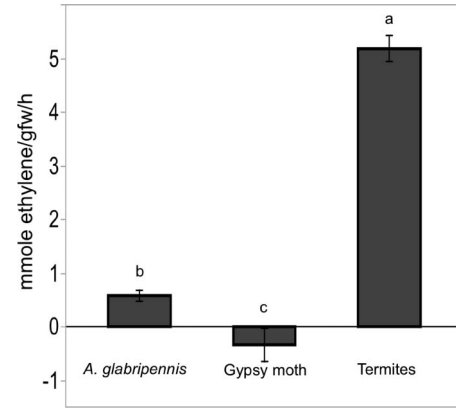

Fig. 4. Acetylene reduction rates (mmole ethylene/g/h) for *A. glabripennis*, termites (*R. flavipes*, positive control), and gypsy moth (*L. dispar*, negative control). Error bars represent the standard error of the mean. ANOVA: $F_{(2, 21)} = 158$; $P < 0.0001$; *A. glabripennis* larvae = 0.58 ± 0.23 (mean ± SEM) ($n = 6$), negative control = −0.33 ± 0.23 ($n = 2$), positive control = 5.17 ± 0.23 ($n = 45$).

occur in a variety of ways; for example, fungi carried in the mycangia of the ambrosia beetle *Dendroctonus frontalis* and inoculated into the phloem of the beetle's host tree, concentrate nitrogen for the insect host (Ayres et al. 2000). Additionally, nitrogen recycling via uric acid hydrolysis is known to occur by the yeast-like symbionts associated with the brown planthopper *Nilaparvata lugens* (Sasaki et al. 1996, Dillon and Dillon 2004). Termites are also capable of obtaining some metabolic nitrogen via nitrogenous waste recycling by bacterial symbionts (Potrikus and Breznak 1980, Dillon and Dillon 2004, Hongoh et al. 2008). Nitrogen recycling by the obligate bacterial symbiont *Blochmannia* has also been reported in carpenter ants (Zientz et al. 2006), as well as by the complementary metabolic pathways of gut microbes and the obligate endosymbiont *Blattabacterium* associated with cockroaches (Sabree et al. 2009).

Similarly, microbial nitrogen fixation has been reported in several insects. This includes gut microbes associated with termites (Potrikus and Breznak 1977), enteric gut microbes in red turpentine beetles (Morales-Jimenez et al. 2013), pine-bark beetles (Ayres et

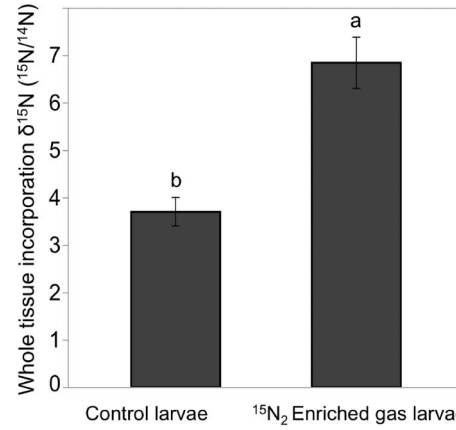

Fig. 5. Bulk ^{15}N ($\delta^{15}N$) incorporated into *A. glabripennis* larvae reared for 2 wk in logs under $^{15}N_2$ gas conditions compared with larvae under $^{14}N_2$ gas conditions in Experiment 1. Results shown are mean values for four replicates per treatment; error bars represent the standard error of the mean and letters represent significantly different group means. ANOVA: $F_{(1,8)} = 12$; $P < 0.014$; $^{15}N_2$ enriched gas larvae = 7.23 ± 0.54 (per mil), control larvae = 4.60 ± 0.54 (per mil; mean ± SEM).

Table 5. Raw $\delta^{15}N$ ($^{15}N/^{14}N$) values for larvae removed from control and treated logs in nitrogen fixation experiment

Experimental group	Sample ID	Instar	$\delta^{15}N$ (‰)
Experiment 1			
Control	N-CT-1-1	Late second instar	5.36
Control	N-CT-2-1	Late third instar	4.02
Control	N-CT-2-2	Late second instar	4.53
Control	N-CT-5-1	Late second instar	4.60
Control	N-CT-5-2	Late third instar	4.52
Control	N-CT-6	Late second instar	3.86
Enriched gas	N-T-1	Early third instar	8.43
Enriched gas	N-T-2-1	Early third instar	7.64
Enriched gas	N-T-2-2	Early third instar	8.75
Enriched gas	N-T-3-1	Early third instar	6.56
Enriched gas	N-T-5-1	Early third instar	5.70
Enriched gas	N-T-5-2	Early third instar	5.47

al. 2000), and stag beetles (Kuranouchi et al. 2006), as well as symbiotic bacteria in the fungus gardens of leaf-cutter ants (Pinto-Tomas et al. 2009) and *Enterobacter sp.* in fruit flies (Behar et al. 2005).

The first documented approach to determine nitrogen fixation is the acetylene reduction assay. This assay relies on similarities in the amount of energy required to reduce the triple bonds in acetylene (C_2H_2) to ethylene (C_2H_4) by an active nitrogenase enzyme complex and to reduce nitrogen to ammonia (946 KJ/mol and 839 KJ/mol, respectively; Hardy et al. 1968). More definitive evidence of nitrogen fixation includes a combination of direct quantification of nitrogen fixation and incorporation using the stable nitrogen isotope ^{15}N, in addition to the acetylene reduction assay and molecular approaches investigating the presence of the genes and transcripts of the nitrogen fixation *nif* regulon in the insect of study (Robinson 2001, Nardi et al. 2002).

Our results support a mechanism by which *A. glabripennis* larvae overcome at least some of its nitrogen limitations in its dietary substrate (wood) through contributions from the gut microbes that fix nitrogen and recycle nitrogenous waste products through urea hydrolysis, making nitrogen available for growth and development. The verification of the presence of urea-hydrolyzing bacteria and nitrogen-fixing bacteria in eggs and postembryonic developmental stages of *A. glabripennis* through the detection of *ureC* and *nifH* genes from DNA samples (Tables 1 and 2), as well as the detection of *ureC* and *nifH* transcripts from RNA samples (Tables 1 and 2) suggest a high level of fidelity of association between urea-hydrolyzing and nitrogen-fixing bacteria with *A. glabripennis*' immature stages. The detection of microbial *ureC* and *nifH* genes and transcripts in surface-sterilized eggs also suggests the possibility of vertical transmission of urea-hydrolyzing and nitrogen-fixing bacteria.

These results are consistent with another study that detected the presence of microbial urease and of *nifH* genes from the *A. glabripennis* larval gut microbial community metagenome (Scully et al. 2013). Furthermore, insect endogenous urease and nitrogenase transcripts were not detected in the larval gut of this species (Scully et al. 2013). Results from *A. glabripennis* are also consistent with studies that detected the presence of microbial *ureC* genes in the eggs of the carpenter ant *Camponotus floridanus*, which harbors the obligate urea-hydrolyzing endosymbiont *Blochmannia* (Zientz et al. 2006). Previous studies on *A. glabripennis* have shown that the beetle larvae harbor a diverse gut microbial community (Geib et al. 2009, Scully et al. 2013), including members of the family Enterobacteriaceae, which consist of several nitrogen-fixing and urea-recycling members (in the examples listed above), and these taxa may be contributors to the observed nitrogen cycling in *A. glabripennis* larvae. Culture-dependent studies to identify some of these taxa more specifically are in progress.

In an attempt to confirm the biological relevance of the presence of urea-hydrolyzing and nitrogen-fixing members of the *A. glabripennis* larval gut community, functional assays employing ^{15}N-stable isotope techniques were used to verify the connection between microbial processes and insect metabolism. Insect tissue incorporation of recycled nitrogen from urea hydrolysis into larval biomass (Fig. 1) and tissue incorporation of fixed nitrogen into larval biomass (Fig. 5) indicate that gut bacteria contribute to larval nitrogen utilization. The link between the microbial ^{15}N-processes in this study and larval ^{15}N-incorporation is proposed to be the GS–GOGAT enzyme complex, which is known to be similarly responsible for the incorporation of recycled or fixed nitrogen in other insect–microbe associations (Zientz et al. 2006, Pinto-Tomas et al. 2009, Sabree et al. 2009, Macdonald et al. 2012).

The specific routing of incorporated recycled nitrogen into nonessential amino acids was significantly greater than into essential amino acids (Fig. 2). The greater ^{15}N-enrichments of nonessential amino acids may be due to more transamination reactions involving the recycled $^{15}NH_2$ and other nonessential amino acids relative to essential amino acids in these larvae. This difference in enrichment may indicate greater use of nonessential amino acids for metabolic processes relative to essential amino acids, coupled with the ability of the insect to synthesize nonessential amino acids de novo (Reeds 2000). For example, we observed more ^{15}N-enrichment of the nonessential amino acid proline in this study (Fig. 3a). The proline–alanine cycle partially oxidizes proline to alanine in the hemolymph, resulting in the formation of 14 molecules of ATP to meet energy requirements (Gäde and Auerswald 2002). The utilization of proline as an energy substrate by both flying and flightless beetles and a number of different insects is well documented (Gäde and Auerswald 2002). The proposed oxidation of proline to alanine is also supported by the observed greater ^{15}N-enrichment of alanine relative to proline and to the other nonessential amino acids (Fig. 3a). All essential amino acids except Lys and Phe were ^{15}N-enriched relative to the artificial diet essential amino acids (Fig. 3b). It is possible that Lys and Phe are not as metabolically active (involved in transamination reactions) as the other essential amino acids.

If we compare the ratio of EAA: NEAA in *A. glabripennis* with the calculated ratios in the pea aphid, *Acyrthosiphon pisum*, which harbors the obligate primary symbiont *Buchnera*, the percentage of nonessential and essential amino acids in aphids were reported as 65 and 36.3%, respectively (Prosser and Douglas 1991), yielding a ratio of 1: 2 EAA: NEAA. The ratio obtained for larvae in our study based on total ^{15}N-enrichment was 1: 6. Thus, in both the pea aphid and in *A. glabripennis* as reported here, the NEAA component of total quantified amino acids was greater than the EAA component. Several studies have examined which individual amino acids are indispensable or essential to insect physiology (Prosser and Douglas 1991, Liadouze et al. 1995); however, it is difficult to determine what the precise ratio of EAA: NEAA is or should be at any point in time due to the combined effects of amino acid turnover (Liadouze et al. 1995), the physiological state of the insect (Prosser and Douglas 1991, Wang et al. 2010), and the quality of the diet (Liadouze et al. 1995).

Overall, this study shows that gut microbial associates of *A. glabripennis* can fix atmospheric nitrogen and hydrolyze urea, contributing to the nitrogen (protein) ecology and thus enabling the insect to overcome nitrogen limitations associated with its diet. This is the first study to our knowledge, to experimentally establish a metabolic connection between gut microbial urea hydrolysis and nitrogen-fixation processes with the physiology of a wood-feeding insect using stable isotopes. This study also adds to the growing body of work investigating the beneficial functions of gut microbial associates in relation to the nutritional ecology of their insect hosts.

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