Insect-Symbiont Interactions

15N Discrimination and the Sensitivity of Nitrogen Fixation to Changes in Dietary Nitrogen in Reticulitermes flavipes (Isoptera: Rhinotermitidae)

MEGAN E. MEUTI,1,2 SUSAN C. JONES,1 AND PETER S. CURTIS3


ABSTRACT  Xylophagous termites possess symbiotic bacteria that fix atmospheric nitrogen (N2). Although symbiotic N2 fixation is central to termite nutrition and ecologically important, it is energetically costly. Using stable isotopes, we tested the hypothesis that symbiotic N2 fixation would decrease in workers of the eastern subterranean termite, Reticulitermes flavipes Kollar, which were exposed to high nitrogen diets. To calculate the isotope discrimination factor occurring as a result of digestion, Δdig, and which occurs as the result of N2 fixation, Δfix, symbiotic N2 fixation was inhibited via force feeding termites the antibiotic kanamycin. Antibiotic-treated termites and control (N2-fixing) termites were exposed to different concentrations of dietary N (0, 0.21, and 0.94% N), their 15N signatures were obtained, and the percent nitrogen derived from the atmosphere within termite samples was calculated. As we hypothesized, symbiotic N2 fixation rates were negatively correlated with dietary N, suggesting that high concentrations of dietary N suppressed symbiotic N2 fixation in R. flavipes. A comparison of the 15N isotope signatures of antibiotic-treated termites with their food sources demonstrated that Δdig = 2.284‰, and a comparison of the 15N isotope signatures of antibiotic-treated termites with control termites indicated that Δfix = −1.238‰. These are the first estimates of Δdig for R. flavipes, and the first estimate of Δfix for any N2-fixing termite species.

KEY WORDS  stable isotope ratios, isotope discrimination factor, atmospheric nitrogen, termites

All organisms require nitrogen (N) for protein and nucleic acid synthesis, yet N is often a limiting nutrient in terrestrial ecosystems. While N limitations can be attributed to several factors, the most critical is the difficulty associated with transforming atmospheric nitrogen, N2, to biologically active forms (Vitousek et al. 1994). Although N2 can be fixed abiotically through lightning, natural fires, and volcanic activity, the vast majority of N2 is fixed by prokaryotic bacteria and Archaea. This process can require as many as 42 molecules of ATP to fix one molecule of N2 in situ (Slavtay 2000). While some N2-fixing microbes exist as free-living organisms, many form obligate or facultative symbioses with algae, higher plants, or animals. Under conditions where N is limiting, organisms that form these symbioses, such as xylophagous termites, are able to dominate their environments (Nardi et al. 2002).

Termites are a diverse group of hemimetabolous insects that consume a variety of woody materials that are rich in cellulose and lignin but poor in nutrients, most notably N. The decay-free wood upon which several species of termites thrive contains as little as 0.03–0.7% N (Tayasu et al. 1994). As termites are 10% dry weight (Holt and Lepage 2000), it is not surprising that termites possess bacteria capable of fixing N2.

In the early 1970s, symbiotic N2 fixation was demonstrated in termites using the acetylene reduction (AR) assay (Benemann 1973, Breznak et al. 1973). Symbiotic N2 fixation has since been found in all families of termites and all feeding types except soil feeders (Holt and Lepage 2000). While AR is able to both demonstrate and quantify symbiotic N2 fixation, there are several shortcomings associated with the assay. These include the rapid suppression of AR rates after termite disturbance, as well as variation in rates depending on oxygen concentrations in the assay vials (Breznak 2000; Curtis and Waller 1995, 1996). These factors likely lead to the underestimation of symbiotic N2 fixation.

A more accurate alternative to AR for assessing symbiotic N2 fixation uses the stable 15N isotope signatures (δ15N) of termites and of their food. Tayasu et al. (1994) employed stable isotopes to assess N2 fixation within termites, deriving the equation:

\[ \% N_{fix} = \frac{(\delta^{15}N_{wood} + \Delta_{dig}) - \delta^{15}N_{termite}}{(\delta^{15}N_{wood} + \Delta_{dig}) - \Delta_{fix}} \times 100 \]
where % N_{dfa} represents the percent N within a termite derived from the atmosphere, and is an indirect measure of symbiotic N₂ fixation; δ¹⁵N_{termite} is the termite’s ¹⁵N isotopic ratio and δ¹⁵N_{wood} is the food source’s ¹⁵N isotopic ratio, both directly measured using an isotope ratio mass spectrometer (IRMS). The discrimination factor occurring as a result of digestion, Δ_{dig} is equal to δ¹⁵N_{termite} – δ¹⁵N_{wood}; and Δ_{fix} is the discrimination factor occurring during symbiotic N₂ fixation. Although neither Δ_{dig} nor Δ_{fix}, were calculated for any N₂-fixing termite species, Tayasu et al. (1994) suggested that 30–60% of N within Neotermes koshunensis Shiraki (Kalotermitidae) was derived from the atmosphere through symbiotic N₂ fixation.

To date, nutritional analyses using stable isotopes have focused mainly on the higher termites, family Termitidae, including litter-feeding and grass-harvesting termites in Asia and Australia (Holt and Lepage 2000, Cook and Dawes-Gromadzki 2005) and fungus growers in Africa (Tayasu et al. 2002). Therefore, little is known about the stable isotope signatures of the Rhinotermitidae, which are xylophagous subterranean termites.

Our objectives were to calculate Δ_{dig} and Δ_{fix} for the eastern subterranean termite, Reticulitermes flavipes Kollar (Rhinotermitidae), and to determine whether the N concentration in food influences symbiotic N₂ fixation in this species. We hypothesized that as dietary N increased, termite dependence on fixed N₂ would decrease, reflecting the high energy costs associated with symbiotic N₂ fixation. Alternatively, low levels of dietary N may stimulate N₂ fixation, again resulting in a negative correlation between dietary N and symbiotic N₂ fixation.

Materials and Methods

Test Organism. Workers of the eastern subterranean termite, R. flavipes, were used in this study. They originated from a single inbred colony that had been established during spring 2002 by pairing a single male and female de-alate; the colony was maintained under established during spring 2002 by pairing a single male and female de-alate; the colony was maintained under

Antibiotic Treatment. An aminoglycoside antibiotic kanamycin (kan) was used to inhibit symbiotic N₂ fixation (Matsuura 2001). This class of antibiotics was selected in part because they bind to the 30S subunit of bacterial ribosomes to inhibit protein production and hence do not affect the eukaryotic protozoa inhabiting the termite hindgut. Furthermore, kan is effective in removing Enterobacter agglomerans (Brenner et al. 1984) and Citrobacter freundii (Schauer and Falkow 1993), the two main N₂-fixing species of bacteria in the termite hindgut.

Forty termites in each of three replicates were individually force fed 100 nl of either 1,000 ppm kan solution (kan⁺) or water (kan⁻ [control]) using a Ultramicropump microapplicator (World Precision Instruments, Sarasota, FL) fitted with a 10 µl flexifil tapertip syringe. A dye that stains cell nuclei, Nile Blue (7.07 mM), served as a visual aid to assess termite consumption of the liquid droplet.

The force-feeding procedure involved restraining an individual termite with an aspirator fitted with a 1–200 µl micropipette tip that was placed between the termite’s head and thorax. Using the microapplicator, the termite then was provided with a 100 nL droplet of the kan solution or water (control) dispensed at its maxillary palps. A dissecting microscope was then used to observe each termite until it had completely ingested the droplet. Force-fed termites were then held together for 2 wk in an enclosed plastic container (5 cm diameter × 3.5 cm height) with a moist cellulose pad.

To assess the efficacy of the antibiotic treatment, symbiotic N₂ fixation rates in termites were measured using AR as described by Bentley (1984). Approximately 20 termites per replicate were placed in a 7 ml glass vial and 1 ml of headspace was replaced with acetylene gas resulting in a final atmosphere of 14% acetylene. These vials were incubated at 25–30°C for 3–4 h, and the headspace gas was analyzed using a Varian CP-3800 gas chromatograph equipped with an FID and HaysepR packed column (Varian, Walnut Creek, CA).

Nitrogen Diets. To assess the effects of dietary N on symbiotic N₂ fixation, termites were fed cellulose pads treated with different N concentrations derived from an ammonium nitrate salt. Ammonium nitrate was chosen as a N source because it has been shown to inhibit N₂ fixation at high concentrations (Curtis and Waller 1997). Distilled water provided a 0% N diet and ammonium nitrate solutions provided 0.21 and 0.94% N diets. All solutions contained Nile Blue dye (7.07 mM) to visually confirm feeding by termites. Cellulose pads were oven dried and preweighed to allow us to subsequently calculate consumption by termites. Each N diet was replicated 10 times, with 28 termites per replicate. Within each N diet, termites from each of five replicates received the kanamycin (kan⁺) or control (kan⁻) force-feeding treatment. Termites were examined every 24–48 h, and dead termites were counted then removed to limit cannibalism. Trophalaxis, the exchange of nutrients between individuals, is a dominant aspect of termites’ digestive physiology (Noirot and Noirot-Timothee 1969), so we made no attempt to limit this process during our study.

At the end of the 2-wk feeding period, the remaining food substrate was oven dried, weighed, and the amount consumed by termites was calculated. For each group of 28 initial termites, cannibalism was estimated by accounting for the total number of dead termites that had been removed and the total number of survivors. Surviving termites had blue thoraxes and abdomens, indicating that they had consumed their respective N diets. The termites’ ability to fix N₂ was assessed using AR, and then they were immediately frozen and subsequently dried at 60°C for 48–72 h.

Termites and representative food samples were analyzed for δ¹⁵N with a Finnigan MAT Delta Plus isotope ratio mass spectrometer (IRMS) interfaced to a Carlo Erba NC2500 elemental analyzer at the Cornell
University Stable Isotope Laboratory (Ithaca, NY). Additionally, the $\delta^{15}\text{N}$ signature of the 0.21 and 0.94% N diets were calculated by measuring the $\delta^{15}\text{N}$ of the ammonium nitrate salt used in their preparation, which was 1.5 ± 0.04‰. These food analyses were conducted using a SIRA Series II IRMS (VG ISOGAS, Middlewich, United Kingdom) at the University of Michigan Field Biological Station (Pellston, MI).

The stable $^{15}\text{N}$ isotope signatures of the antibiotic-treated termites ($\delta^{15}\text{N}_{\text{kan-termites}}$) and the control termites ($\delta^{15}\text{N}_{\text{kan-termites}}$) and their diet ($\delta^{15}\text{N}_{\text{food}}$) were used to calculate $\Delta_{\text{dig}}$ and $\Delta_{\text{fix}}$ according to the following equations:

$$\Delta_{\text{dig}} = \delta^{15}\text{N}_{\text{kan-termites}} - \delta^{15}\text{N}_{\text{food}}$$

$$\Delta_{\text{fix}} = \delta^{15}\text{N}_{\text{kan-termites}} - \delta^{15}\text{N}_{\text{kan-termites}}$$

These data allowed us to determine percent nitrogen derived from the atmosphere ($\text{N}_{\text{dfa}}$) within control termites based on the equation derived by Tayasu et al. (1994).

**Data Analyses.** All treatment effects were evaluated using two-tailed homoscedastic (equal variance) Student’s t-test. One way analysis of variance (ANOVA) was used to assess differences in mortality and cannibalism in kan$^+$ or kan$^-$ termites among the different N diets after the arcsine square-root transformation for percentage data. One way ANOVA was used to evaluate the correlation between dietary N and symbiotic N$_2$ fixation among termites exposed to the three N diets. Tukey’s pairwise comparisons were also used to evaluate the effects of dietary N on symbiotic N$_2$ fixation. An $\alpha$-level of 0.05 was used in the above tests.

**Results**

**Antibiotic Treatment.** Symbiotic N$_2$ fixation in antibiotic-treated termites was reduced below equipment detection levels and was significantly less than that of control termites ($t_2 = 7.21; P < 0.0001$). No significant differences in mortality were observed between kan$^+$ and kan$^-$ termites exposed to any of the N diets (16.07 versus 16.43% mortality for the 0% N diet, 46.43 versus 46.43 for the 0.21% N diet, and 53.57 versus 46.43 for the 0.94% N diet). Additionally, there were no significant differences in presumed cannibalism for kan$^+$ and kan$^-$ termites exposed to different N diets.

**$^{15}\text{N}$ Signatures of Termites and Calculation of $\Delta_{\text{dig}}$ and $\Delta_{\text{fix}}$.** The kan$^+$ and the kan$^-$ termites consumed similar amounts of the 0.21 and 0.94% N diets, and both of these diets had the same $\delta^{15}\text{N}$ signatures (Fig. 1a). What is noteworthy is that the $\delta^{15}\text{N}$ signatures of the kan$^+$ termites were significantly higher than those of the kan$^-$ termites on the same diet (0% N diet; $t = 9.25$, df = 8, $P < 0.0001$; 0.21% N diet; $t = 4.09$, df = 6, $P = 0.006$; 0.94% N diet; $t = 2.84$, df = 6, $P = 0.029$) (Fig. 1a). This demonstrates that the antibiotic treatment had an effect on the overall termite $\delta^{15}\text{N}$ signature, which is crucial for our calculation of $\Delta_{\text{dig}}$ and $\Delta_{\text{fix}}$. Moreover, the $\delta^{15}\text{N}$ signature of kan$^+$ termites increased as dietary N increased ($t = 11.2$, df = 6; $P < 0.001$) (Fig. 1a).

These trends are further reflected in Fig. 1b which shows calculated values of the discrimination factors $\Delta_{\text{dig}}$ and $\Delta_{\text{fix}}$. As expected, $\Delta_{\text{dig}}$ did not significantly change as dietary N increased from 0.21 to 0.94%, and we therefore averaged the two calculated values to obtain an estimate that $\Delta_{\text{dig}} = 2.284 ± 0.061‰$. We found that $\Delta_{\text{fix}}$ was most negative, indicating the highest symbiotic N$_2$ fixation, for termites exposed to the 0% N diet and that $\Delta_{\text{fix}}$ became increasingly less negative as dietary N increased (Fig. 1b). As we hypothesized, symbiotic N$_2$ fixation was inhibited in termites exposed to the 0.21 and 0.94% N diets (Fig. 1) and hence the $\Delta_{\text{fix}}$ value for termites exposed to the 0% N diet ($-1.238 ± 0.135$) was used in subsequent calculations of the %N$_{\text{dfa}}$ in termite samples.

**Dietary Nitrogen Effects on Symbiotic N$_2$ Fixation.** Termites that lacked N in their diet had high rates of N$_2$ fixation as evidenced by >60% of their tissue N being derived from the atmosphere (Fig. 2). Furthermore, symbiotic N$_2$ fixation was significantly different.
among the dietary N treatments (F = 37.4; df = 2, 11; P < 0.001). A Tukey’s pairwise comparison indicated that as dietary N increased, the %N\textsubscript{dfa} within the termites decreased (0 and 0.21% N diets: t = 3.52, df = 8, F = 0.0079; 0.21 and 0.94% N diets: t = 13.7, df = 8, P < 0.001). Therefore dietary N and symbiotic N\textsubscript{2} fixation were negatively correlated.

### Discussion

Although the estimation of Δ\textsubscript{dig} and Δ\textsubscript{fix} has posed a challenge for using stable 15N isotopes to assess symbiotic N\textsubscript{2} fixation in the past (Waller 2000), we overcame this difficulty.

Using experimentally derived values for these two isotope discrimination factors, we found that symbiotic N\textsubscript{2} fixation rates for \emph{R. flavipes} were responsive to changes in dietary N. As we hypothesized, the high energy cost associated with symbiotic N\textsubscript{2} fixation was reflected in a negative correlation with dietary N in termites. Even though antibiotic-treated and control groups consumed similar amounts of food, the δ\textsuperscript{15}N signatures of kan\textsuperscript{-} termites became more similar to kan\textsuperscript{+} termites as dietary N increased (Fig. 1a). This suggests that removing the N\textsubscript{2}-fixing bacteria (kan\textsuperscript{-}) caused termites to have a δ\textsuperscript{15}N signature that was more similar to their food source, whereas termites that maintained their N\textsubscript{2}-fixing symbionts (kan\textsuperscript{+}) had a lower δ\textsuperscript{15}N signature than their food. Therefore, increasing concentrations of dietary N had an approximately equivalent effect as the antibiotic treatment. That is, high concentrations of dietary N as well as antibiotic treatment inhibited symbiotic N\textsubscript{2} fixation in termites.

The reduction in symbiotic N\textsubscript{2} fixation rates we observed after termites had fed on substrates containing 0.21 and 0.94% N is biologically relevant as our previous analyses have shown that pine (\emph{Pinus} spp.) wood and needles, and aspen leaves (\emph{Populus grandidentata} Michaux), which are substrates that termites naturally encounter, contained 0.07, 0.34, and 0.94% N, respectively (Meuti, unpublished data). Furthermore, because termites in our study were allowed to engage in trophallaxis, it is likely that they were actively exchanging materials that were richer in nitrogen than the 0, 0.21, and 0.94% N food sources to which they were experimentally exposed. Nonetheless, despite this phenomenon, we observed a negative correlation between dietary N and symbiotic N\textsubscript{2} fixation.

Our results contradict the inference made by Curtis and Waller (1997) that it is unlikely that N concentrations in natural food substrates are high enough to influence symbiotic N\textsubscript{2} fixation. Their assessment was based on AR results that demonstrated a significant decrease in N\textsubscript{2} fixation only after \emph{Reticulitermes} spp. had fed on a very high N diet (5% N derived from ammonium nitrate). In contrast, our data indicated that food sources with as little as ~0.2% N significantly reduced N\textsubscript{2} fixation rates. The inherent sensitivities of our different assays could account for these disparate conclusions. Stable 15N isotopes are more sensitive than AR to changes in N\textsubscript{2} fixation rates, and hence we suggest that they should be favored over AR in future studies of symbiotic N\textsubscript{2} fixation in termites and other terrestrial arthropods.

Previous studies on symbiotic N\textsubscript{2} fixation in termites have assumed that Δ\textsubscript{fix} is within the range established for nodulating plants (−2 and 0‰) (i.e., Tayasu et al. 1994). Our methodology of eliminating symbiotic N\textsubscript{2} fixation through force feeding the antibiotic kanamycin to termites produced an estimate of Δ\textsubscript{fix} = −1.238‰, which suggests that Δ\textsubscript{fix} values may be widely conserved across plants and arthropods that possess N\textsubscript{2}-fixing symbionts.

Our estimate of Δ\textsubscript{dig} = 2.284‰ is consistent with values reported for some termite species (Tayasu 1998). Other studies that calculated symbiotic N\textsubscript{2} fixation in termites (Tayasu et al. 1994, Cook and Dawes-Gromadzki 2005) have estimated Δ\textsubscript{dig} according to the “universal” 15N isotopic enrichment factor, assumed to
be 3.4‰ (Minagawa and Wada 1984). However, $\Delta_{\text{dig}}$ values can vary according to the species being studied, the individual’s age, the form of N excretion, the tissues being examined, as well as the $\delta^{15}$N signature of the diet (DeNiro and Epstein 1981, Vanderklift and Ponsard 2003, Overmann and Parrish 2001, Caut et al. 2008). Moreover, a meta-analysis of 134 different estimates of $\delta^{15}$N isotopic enrichment conducted by Vanderklift and Ponsard (2003) provided an overall mean estimate of $\Delta_{\text{dig}} = 2.54\%$e, with a value of 2.73‰ for uricotelic organisms, such as termites. These data suggest that there is no “universal” $\Delta_{\text{dig}}$ and hence the assumed value of 3.4‰e should not be used in future studies.

Studies by Vanderklift and Ponsard (2003) and Robbins et al. (2005) further suggested that $\Delta_{\text{dig}}$ may be negatively correlated with protein quality as measured by the C:N ratio. However, Minagawa and Wada (1984) suggested just the opposite given that $\Delta_{\text{dig}}$ increased with higher trophic levels and was positively correlated with a higher quality N supply. Clearly the relationship between $\Delta_{\text{dig}}$ and dietary N warrants further study. Our observation that $\Delta_{\text{dig}}$ did not significantly change in response to increasing dietary N could be a result of the low variation of the %N and C:N ratios in our termite diets, or perhaps the $\delta^{15}$N signature of the food may exert a greater effect than dietary N content as suggested by Caut et al. (2008).

We observed significant differences between the $\delta^{15}$N signatures of kan+ and kan- termites that had been feeding for 2 wk on a $-1.5\%$e N food source, yet it is possible that the $\delta^{15}$N signatures of termites may have had insufficient time to stabilize. The termites’ $\delta^{15}$N signatures possibly could diverge further if termites were allowed to feed for a longer period of time.

As nitrogen recycling and the form of nitrogen excretion are thought to strongly affect $\Delta_{\text{dig}}$ (Vanderklift and Ponsard 2003), the potential effects of kanamycin treatment on $\Delta_{\text{dig}}$ and the termites’ ureolytic bacteria are considerations that warrant further investigation. However, the termite hindgut is mostly anaerobic and the antibiotic kanamycin is most effective against aerobic gram-negative bacteria. Therefore, antibiotic treatment presumably should not have affected the ureolytic bacteria in the termite hindgut.

While recent work demonstrates the variability and mutability of $\Delta_{\text{dig}}$ in response to several factors, we are gaining a better understanding of this term and new models have increasing predictive value (Caut et al. 2008). Therefore, modified estimates, as well as our own calculated value of $\Delta_{\text{dig}}$ should allow researchers to accurately determine food preferences and feeding substrates of R. flavigaster in their natural habitat.

Although our results were obtained using hundreds of termites from a single colony that thrived for 6 yr in the laboratory, future research should be conducted using multiple colonies, including field-collected termites. Research with such colonies is anticipated to support our results, and it also will allow determination of the amount of fixed N$_2$ that R. flavigaster contributes to terrestrial environments. Such research will allow us to better assess this termite’s environmental importance and its role as an ecological keystone species.

Future research should also address whether other N sources inhibit symbiotic N$_2$ fixation, particularly as high concentrations of the ammonium ion, NH$_4^+$, are known to inhibit the transcription of the nitrogenase enzyme which breaks down atmospheric nitrogen (Hoover 2000). In preliminary studies, we observed a significant reduction in symbiotic N$_2$ fixation (~9% N$_{\text{fix}}$) after termites had consumed 0.94% N derived from urea (Meuti, unpublished data). In contrast, Curtis and Waller (1997) observed no effects on symbiotic N$_2$ fixation after termites consumed 5% N derived from urea. We furthermore observed that symbiotic N$_2$ fixation decreased when termites consumed natural substrates (pine wood, pine needles, and aspen leaves) that had increasing levels of dietary N. Therefore, we anticipate that future studies on field-collected termites will support our finding that symbiotic N$_2$ fixation is negatively correlated with increasing concentrations of dietary N, independent of its source.

The previous lack of calculated values for $\Delta_{\text{dig}}$ and $\Delta_{\text{fix}}$ has been a pitfall of using stable isotopes to assess symbiotic N$_2$ fixation (Waller 2000). As the calculation of %N$_{\text{fix}}$ in termite samples is incredibly sensitive to slight changes in either $\Delta_{\text{dig}}$ or $\Delta_{\text{fix}}$, the use of general estimates for these values is problematic. Our methodology of force feeding an antibiotic to each individual termite produced tenable estimates of important metabolic discrimination factors whereas previous studies simply relied on estimates from mammalian and plant systems. Our study is the first to calculate $\Delta_{\text{dig}}$ for R. flavigaster and also to calculate $\Delta_{\text{fix}}$ for a N$_2$-fixing termite, hence contributing to our understanding of symbiotic N$_2$ fixation in this species. While our result that $\Delta_{\text{fix}} = -1.238\%$e is within the range previously established for nodulating plants, our calculation of $\Delta_{\text{fix}} = 2.284\%$e differed greatly from the “universal” value of 3.4‰e used in other studies on symbiotic N$_2$ fixation in termites (Tayasu et al. 1994, Cook and Dawes-Gromadzki 2005). The methodology we used and the results we obtained should have interesting implications for future research on N$_2$ fixation in other termite and arthropod species.

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