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Factors Affecting Nitrogen Fixation Rates in Termites (Isoptera: Rhinotermitidae: *Reticulitermes*) and Termite Nitrogen Contributions of Forest Ecosystems

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FACTORS AFFECTING NITROGEN FIXATION RATES IN TERMITES (ISOPTERA: RHINOTERMITIDAE: *RETICULITERMES*) AND TERMITE NITROGEN CONTRIBUTIONS TO FOREST ECOSYSTEMS

by

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ABSTRACT

FACTORS AFFECTING NITROGEN FIXATION RATES IN TERMITES (ISOPTERA: RHINOTERMITIDAE: RETICULITERMES) AND TERMITE NITROGEN CONTRIBUTIONS TO FOREST ECOSYSTEMS

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Termites host symbiotic bacteria that fix atmospheric nitrogen, which makes termites important in the biogeochemical cycling of nitrogen in ecosystems. The objective of this study was to estimate annual nitrogen contributions by termites by examining seasonal rates of nitrogen fixation, seasonal abundance of different termite castes and their nitrogenase activity, the effects of dietary nitrogen on nitrogenase activity, and low oxygen and high carbon dioxide atmospheres on termite nitrogenase activity, and the accuracy of mark-recapture studies to estimate termite population size.

Termite nitrogen fixation rates were monitored monthly for 10 *Reticulitermes* Holmgren colonies from June, 1993, to December, 1995. Rates were highest in fall and spring and lowest in the winter and summer. Workers fixed more nitrogen than other castes and were the most abundant caste.

Nitrogenase activity in other nitrogen fixation systems is influenced by dietary nitrogen. The nitrogen content of natural termite food was not correlated with nitrogenase activity in the field, but nitrogenase activity was suppressed in termites on diets enriched with certain types of nitrogen.

Nitrogen fixation in other nitrogen fixing systems is oxygen sensitive. Termites foraging within logs and deep in the soil may be exposed to low oxygen and high carbon

dioxide conditions. In laboratory experiments, termite nitrogenase activity increased with decreasing oxygen concentration. In contrast, increased carbon dioxide concentrations did not affect termite nitrogenase activity.

Although previous studies indicate that termite nitrogenase activity decreases in the laboratory, in this study termites maintained or exceeded their original rates in the laboratory. Initial environmental conditions in the field affected the ability of termites to regain or exceed their original nitrogen fixation rates.

Mark-release-recapture experiments were conducted in the laboratory to test the accuracy of the Lincoln index used to estimate termite populations. Population estimates for termite colonies fed 0.1% (w/w) Nile blue were approximately 3 times greater than the actual population size while they were 10 times greater for termites fed 0.05% (w/w). Potential model biases are discussed in reference to mark-release-recapture in termites fed ingestible dye markers.

Based on this study and previous termite abundance studies, *Reticulitermes* is capable of contributing 76.84 - 384.21 g Nitrogen • ha^{-1} • yr^{-1} to temperate forest ecosystems.

To the late John Stuart Curtis, your loving memory will from my mind never fade.

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CHAPTER I

INTRODUCTION[1]

There are seven families and approximately 2500 species of termites in the order Isoptera worldwide (Waller and La Fage 1987a). All are eusocial and colonies are composed of distinct castes which include workers, soldiers, nymphs, and larvae (Krishna 1989). The workers are usually the most abundant caste at any given time of the year (Baroni-Urbani *et al.* 1978). They are responsible for most of the colony maintenance (i.e., feeding of the dependent castes, foraging, structural maintenance) (Krishna 1989). Soldiers serve to protect the colony from invasion by predators (Krishna 1989). Nymphs are prealate forms which function as reproductive stock for the colony. Larvae are the undifferentiated young of the reproductives, and they usually represent the second largest in colony caste proportion (Howard and Haverty 1981).

The Isoptera are phylogenetically separated into lower termites (Mastotermitidae, Kalotermitidae, Hodotermitidae, Termopsidae, Rhinotermitidae, and Serritermitidae) and the higher termites (Termitidae). Termites are closely related to cockroaches, and they are thought to have evolved from a primitive cockroach-like ancestor approximately 200 million years ago (Krishna 1989).

Termites can also be grouped into four general ecological types (Nutting and Jones 1990) which are based on where they live and the food they consume. The ecological

[1]The *Annals of the Entomological Society of America* was used as the journal model for the placement of figure titles, table titles, and the format of the reference section.

groupings of termites are: dry wood, damp wood, harvester, and subterranean. Dry wood termites are not dependent on the ground for moisture. Their source of water is derived metabolically and they are usually found in posts, stumps, and sometimes buildings made of wood (Krishna 1989). Dampwood termites also do not require soil contact for moisture, but they do require moisture in the wood they consume. They are found in dead, damp, and rotten logs (Krishna 1989). The term harvester is associated with termites that store food products, or harvest fungus which grows in termite galleries. The amount of food stored is reported to vary from one night's forage (Darlington 1982) to stores lasting up to two weeks (Collins 1981). Colonies of subterranean termites must have contact with the soil in order to survive (Krishna 1989). Subterranean termites are by far the most destructive to human carpentry endeavors, costing billions of dollars annually for damage repair and treatment (Su and Scheffrahn 1990). Once the soil nest is established, termites may enter unprotected wooden structures through contact with the soil, cracks in masonry, or by tubes constructed from soil and glandular secretions. Damage to the affected wood may be great, and the structure may crumble or collapse entirely. Termite infestation could mean financial ruin for homeowners. On the other hand, it is a very favorable condition for forest ecosystems.

Termites and Biogeochemical Cycling

In addition to being an economically important pest, termites are also important ecologically to forest ecosystems. They are closely linked with biogeochemical (nutrient) cycling. The major biogeochemical cycles are: hydrologic, carbon, oxygen, nitrogen,

sulfur, and phosphorus (Staley and Orians 1992). Components of each conceptually belong to "reservoir pools". Depending on the scale, reservoir pools may include all or part of the atmosphere, the ocean, the sediments, and living organisms. In general, flux between reservoirs is dominated by the biota and their activities. Elements are exchanged slowly between some reservoirs over a long period of geological time (Rodhe 1992).

Termites are important in the carbon cycle through their roles as consumers and detritivores (DeAngelis 1992). The termite gut is host to protozoan and bacterial symbionts that are able to digest wood cellulose and thus release the energy otherwise unavailable to the insects (Waller and La Fage 1987a). Termite foraging and tunnelling redistributes soil and increases the surface area available to bacteria and fungi (Wood and Sands 1978). The breakdown of lignin and cellulose found in wood is primarily facilitated by the enzymatic secretions of fungi (Bold *et al.* 1980). Fungi are also able to liberate various elements such as nitrogen, phosphorus, potassium, sulfur, iron, calcium, magnesium, and zinc (Bold *et al.* 1980). The ability of termites to influence the physical structure and chemical nature of their environment impacts vegetation and other components of the ecosystem (Wood and Sands 1978). Their effect on the nitrogen cycle has traditionally been recognized as returning nutrients to the ecosystem. However, recent studies indicate that termites may play a larger role in the cycling of nitrogen than was once thought.

The atmosphere is composed of 78% nitrogen as N_2 gas. This represents a reservoir pool for nitrogen in terrestrial ecosystems. Biota cannot use this element until it is "fixed" into useable forms. Nitrogen fixation most commonly occurs in two ways:

lightning accounts for the fixation of 10% of global available nitrogen and the other 90% of fixed nitrogen is generated from the action of microbes. Free living bacteria, cyanobacteria, and symbiotic bacteria in nodules in the low O_2 environment within heterocysts of leguminous plants are all involved in microbial nitrogen fixation (Jaffe 1992). However, there is another place where nitrogen fixation occurs.

The microbial gut flora of termites include nitrogen fixing bacteria (Benemann 1973, Breznak *et al.* 1973, French *et al.* 1976, Potrikus and Breznak 1977, Prestwich and Bentley 1981). The rate at which nitrogen is fixed varies among termite species (Prestwich *et al.* 1980, Breznak 1984, Bentley 1987, Waller *et al.* 1989) and within species as a function of food quality (Breznak *et al.* 1973), termite caste (Prestwich *et al.* 1980, Hewitt *et al.* 1987), and termite size (Waller *et al.* 1989). Intraspecific variation may also exist due to seasonal factors (Waller *et al.* 1989). Newly fixed nitrogen is incorporated into termite tissue, excretion products, and secretion products (Bentley 1984).

Termites and Forest Ecosystems

Nitrogenous products of termite origin may enter and be distributed within the ecosystem in several ways. Adult termites are able to pass nitrogen-containing compounds to their young by trophallaxis. This transfer can occur from stomodeal food, proctodeal food, and salivary secretions (Waller and La Fage 1987a). Stomodeal food is food that is partially digested in the crop of the donating termite which is regurgitated and fed to a recipient termite. The dependent castes receive nutrients and digestive

enzymes in the process. Although this behavior is not common among termites (La Fage and Nutting 1978), it represents a possible mode of distribution of nitrogen containing compounds to colony members. Proctodeal food is transferred from the anus of donor termites. This food is also partially digested and differs from feces (Waller and La Fage 1987a). Flagellate protozoans and other gut symbionts are transferred along with proctodeal food. Saliva is rich in lipids and protein (La Fage and Nutting 1978) and is also fed to dependent castes (Waller and La Fage 1987a).

Another way in which nitrogen may enter the ecosystem is through direct deposition onto soil. Salivary secretions mixed with soil and wood particles are used by termites to build tunnels and galleries. Most termite nests are made of carton which is composed of soil mixed with termite feces. These galleries, tunnels, and carton can extend far into the soil, and this close association to the soil offers opportunity for the nitrogenous compounds to adsorb to soil components (Wood and Sands 1978).

Finally, further N-distribution into the ecosystem by termites may occur through the seasonal dispersion during reproductive flights of winged adults called alates. Species differ as to the time of dispersal and number of dispersal events. In general, alates develop from workers or nymphs. At the appropriate time they fly away from the colony, form mating pairs, and generate new colonies far from the original nest site (Nutting 1969). Most alates fall victim to predation and other environmental factors. In this way, their complement of nitrogen is recycled in the form of food and detrital material (DeAngelis 1992).

Termites and Soil

Termites are able to change the physical and chemical properties of soil. Tunneling and foraging behaviors loosen, aerate, and redistribute soil particles. The depths at which subterranean termites are able to effect these changes range from the surface to 8.5 meters (Watson 1960). Lee and Wood (1971) reported termites at a depth of 70 meters. Foraging areas have been measured at 266 to 1091 m^2 for *Reticulitermes flavipes* (Kollar) (Grace *et al.* 1989, Grace 1990). Chemical alteration of soil by termites usually results in the increase of pH, calcium, phosphorus, and potassium (Wood and Sands 1978).

The potential dispersion of nitrogenous compounds (in termite tissues, excretia, and secretia) to other termites, other species (i.e., predators, detritivores, decomposers, etc.) and its direct deposition onto soil may make nitrogen fixation in termites an important factor in the nutrient cycles of forest ecosystems. Based on nitrogen fixation rates reported by Pandey *et al.* (1992), *Reticulitermes* spp. are capable of contributing nitrogen in amounts similar to those reported for free-living bacteria of the same habitat (Waide *et al.* 1988). However, estimates based on seasonal data of nitrogenase activity are needed because factors such as seasonal colony dynamics, temperature, food nitrogen content, and atmospheric conditions affect nitrogen fixation.

Seasonal Colony Dynamics

To estimate annual contribution of N per hectare, estimation of colony biomass is essential. Two measurements are necessary for the determination of termite biomass,

1) the number of colonies per hectare and 2) the number of individual termites per colony. Howard *et al.* (1982) reported the number of colonies per hectare for *R. flavipes* and *R. virginicus* was 4.4 and 2.4, respectively. Mark-recapture experiments have shown *R. flavipes* colony size to range from 943,237 $\pm$ 14,133 and 722,697 $\pm$ 189,729 (Grace 1990) to 3,187,538 $\pm$ 606,341 and 2,084,219 $\pm$ 323,049 (Grace *et al.* 1989) in Ontario, Canada. All of these mark-recapture estimates exceed the mean colony size of *Reticulitermes* spp. (244,445) determined by destructive sampling methods used by Howard *et al.* (1982) in Mississippi. There is considerable variation in estimates of colony size. Whether the observed variability is a natural phenomenon related to seasonality or geography, or whether it is the result of sampling methodology is unknown. Laboratory experiments were designed to test the accuracy of a mark-recapture method used by some termite researchers (Southwood 1971).

Nitrogenase activity varies among castes (Breznak 1984, Hewitt *et al.* 1987) and caste composition varies seasonally (Howard and Haverty 1981). Therefore, it is important to determine nitrogen fixation rates for each caste within a colony, coupled with seasonal data on caste ratios. Seasonal variation of caste composition and colony size may affect the amount of nitrogen contributed to forest ecosystems. Previous studies have estimated termite nitrogen contributions without considering seasonal fluctuations in caste composition and colony size. This study provides the first forest-wide estimates of N contributed by termites to forest ecosystems.

Temperature

Nitrogen fixation rates are dependent on temperature. Waller (unpubl.) found that workers of *Reticulitermes* spp. maintained at 25 ^{0}C exhibited significantly more nitrogenase activity than those kept at 30^0 C. This finding suggests that seasonal variation in temperature may contribute to variation in nitrogen fixation rates. Summer temperatures are expected to cause a drop in nitrogenase activity. To test this hypothesis, nitrogenase activity of field foraging groups were determined and the mean temperature was recorded monthly for nearly three years.

Food Nitrogen Content

Foods high in nitrogen suppress nitrogenase activity in termite bacterial symbionts (Breznak *et al.* 1973, Waughman *et al.* 1981). Maudlin and Rich (1975) found that artificial diets of casein and albumin in excess of 5% were toxic to termites. In nature, the nitrogen content of wood may be influenced by fungal decay (Hudson 1972, Collins 1983). Although it is controversial whether termites select high-N foods in nature, the nitrogen content of natural foods may influence the amount of nitrogen fixed by termites. Therefore, a major goal of the study was to relate termite nitrogen fixation rates to the nitrogen content of their food. In addition to seasonal field experiments, nitrogenase activity was also related to termites fed artificial diets enriched with different sources and concentrations of nitrogen in laboratory experiments.

Atmospheric Conditions

Since subterranean termites establish nests within their food (Wood 1978) and often at great depths (Watson 1960), termites may spend most of their time under atmospheric conditions different from those closer to the surface. Carbon dioxide concentrations are likely to be high in termite nests (Peakin and Josens 1978). Matsumoto (1977) found that nests of the Macrotermitinae had a $pCO_2 = 0.052$ atm which is ≈150 times greater than ambient levels. Increased ambient levels of CO_2 in the atmosphere and its role in global warming have been well documented. However, its effect on termite nitrogen fixation has yet to be determined. Laboratory experiments were designed to measure nitrogenase activity in response to increased levels of CO_2.

Symbiotic and asymbiotic nitrogen fixation is inhibited by O_2 (Burris *et al.* 1955, Parker and Scutt 1960, Bergensen 1962). Paim and Beckel (1964) found low oxygen concentrations ($pO_2 = 0.05$ atm) within decayed logs. If conditions are more favorable for nitrogen fixation within termite nests (low pO_2) than the conditions at which the termites are assayed for nitrogenase activity (ambient pO_2), then the estimates of forest-wide nitrogen contribution by termites may be underestimated. Laboratory experiments were designed to measure termite nitrogen fixation rates in response to decreased pO_2.

CHAPTER II

SEASONAL PATTERNS OF NITROGEN FIXATION RATES IN *RETICULITERMES* IN A FOREST ECOSYSTEM

Introduction

The microbial gut flora of termites include nitrogen-fixing bacteria (Breznak et al. 1973, Potrikus and Breznak 1977). The newly fixed nitrogen is incorporated into termite tissues and is distributed throughout the colony (Bentley 1984, Waller and La Fage 1987a). Through this process, termites supplement their nitrogen poor diet and contribute to the biogeochemical cycling of nitrogen in some ecosystems (Tayasu et al. 1994, Slaytor and Chappel 1994, Schaefer and Whitford 1981, Pandey et al. 1992). Termite nitrogen fixation rates have been reported to vary among termite species (Breznak 1982). Intraspecific variation is attributed to differences in food quality, termite caste, termite size, and seasonal factors (Prestwich et al. 1980, Breznak et al. 1973, Waller et al. 1989, Curtis and Waller 1995). Previous studies have demonstrated that nitrogenase activity is higher in soldiers in some termite species and lower in other species (Prestwich *et al.* 1980). Larvae have been reported to fix ≈ 300-fold more nitrogen as workers in *Coptotermes formosanus* Skiraki (Breznak 1982). To understand fully the nitrogen contribution of termites to ecosystems, long-term studies are needed to determine the effects of seasonal factors and colony dynamics.

In the present study, termite worker nitrogen fixation rates were examined monthly

for 31 months in a tidal forest in coastal Virginia. Seasonal caste composition was determined every two months throughout the study period, and the nitrogen fixation rates of the different termites castes were measured when they were available. An estimation of termite nitrogen contribution to forest ecosystems was calculated based on seasonal colony dynamics and termite abundance data from current and previous studies.

Materials and Methods

Termites. Wood infested with *Reticulitermes flavipes* (Kollar) and *Reticulitermes virginicus* (Banks) was collected monthly from the headquarters of the Virginia Coast Reserve (VCR) Long Term Ecological Research (LTER) site located in Nassawadox, on the Eastern Shore of Virginia from June 1993 through December 1995 for the worker assays. Environmental temperature data were obtained from a database maintained by the VCR-LTER (Krovetz and Porter 1993-1995). Termites were also collected from other sites in southeastern Virginia, for worker, soldier, nymph, larva, and alate assays. *R. flavipes* and *R. virginicus* are common to all of our study sites. However, termites were not identified to species when no alates were present. Voucher specimens have been deposited at Old Dominion University.

Acetylene Reduction Assay. The acetylene reduction assay was used to determine nitrogenase activity for each termite colony. The assay is based on the ability of nitrogenase to reduce acetylene (C_2H_2) to ethylene (C_2H_4) at three times the rate it converts dinitrogen (N_2) to ammonia (NH_3) (Hardy *et al.* 1973, Bentley 1984). The following protocol is adapted from Pandey et al. (1992).

Fifty worker termites, removed from their nest material and weighed to the nearest 0.1 mg, were placed in an 8.5-ml glass vial with a rubber sleeve cap septum. One ml of air was removed and 1.0 ml of acetylene was added to the vial, resulting in a final atmosphere of approximately 12% acetylene. Three replicate vials per colony were incubated at 22 $\pm$ 2 C for 30 minutes. Following incubation, a 200-ul sample of head space was removed with a 0.5-ml Hamilton Gas Tight® syringe and injected into a Varian® 3600 gas chromatograph equipped with a flame ionization detector and a Porapak® N column. Ethylene peaks were analyzed using a standard concentration curve with known amounts of ethylene to determine the moles of ethylene produced for each sample. Final nitrogen fixation rates are expressed as dinitrogen fixed (ug)• termite fresh mass $(g)^{-1} \bullet day^{-1}$. There were three replicate samples for each colony. Ten colonies per month were sampled for 31 consecutive months. Nitrogenase activity was also measured for the different castes when they were available.

Caste Proportion. The proportion of different caste members was determined for the same 10 termite colonies collected from the VCR, used in the worker nitrogenase activity assays, every other month from February, 1994 to December, 1995. Termites were removed from their nest material and placed into an 8-mesh wire sieve to remove large debris. The volume of the sifted-material, including termites, was measured. Three replicate samples, each with a volume containing $\approx$ 250 termites, were examined for the presence of the following six castes: worker, soldier, presoldier, nymph, larva, and alate.

Percent Body Water. For the months in which caste ratio was determined, the fresh weight of two replicate samples containing 10 termites of each caste was recorded

for all colonies. The termites were killed by freezing then dried in an oven at 56 $\pm$ 2 C for 48 hours. Dry mass was recorded and percent body water was determined.

Termite Abundance. Estimates of the number of *R. virginicus* in two logs were determined by a mark-release-recapture method using the ingestible dye marker Nile blue. Termites were collected from logs in the field, taken to the laboratory, removed from the wood, and fed filter paper impregnated with 0.1% (w/w) of the dye Nile blue for five days. A week later, marked termites were counted and released to the original host logs. After seven days, a 30 cm section of the log was collected, returned to the laboratory, and broken to expose all termites. The broken pieces were then placed into an 8-mesh wire sifter to remove the wood particles from the termites. Marked and unmarked individuals were counted and population estimates were calulated using the Lincoln index (Southwood 1971).

Statistical Analysis. The monthly nitrogen fixation rates of worker termites were analyzed using a completely randomized design analysis of variance (ANOVA) to test if there was a difference among the 31 months of the study ($\alpha = 0.05$) (SAS statistical package, SAS Institute 1990). The nitrogen fixation rates of each termite caste were analyzed with a completely randomized design analysis of variance (ANOVA) to test for a difference in nitrogenase activity among the different termite castes over the study period ($\alpha = 0.05$) (SAS statistical package, SAS Institute 1990). The fresh weight, dry weight, and percent body water of the termites was analyzed with a completely randomized design analysis of variance (ANOVA) to test for differences over the 31 months of the study ($\alpha = 0.05$) (SAS statistical package, SAS Institute 1990). Caste ratio

data were transformed (arcsin square-root) and analyzed with a completely randomized design analysis of variance (ANOVA) to test for differences in caste proportion for each caste over the study period ($\alpha = 0.05$) (SAS statistical package, SAS Institute 1990). The Tukey honestly significant difference (HSD) multiple comparison test was used to compare means. Termite abundance was determined by mark-release-recapture (Lincoln index) for two colonies at our VCR study site.

Results

Nitrogenase Activity. There was a significant difference among monthly termite nitrogen fixation rates for the 31 months of the study ($F = 32.92$; df = 30, 887; $P < 0.0001$) (Table 1) (Fig. 1). These differences were related to seasonal variation in nitrogenase activity ($F = 23.76$; df = 3, 226; $P < 0.0001$) with the highest rates occurring in the moderate temperatures of spring and fall and the lowest rates occurring in the extreme temperatures of winter and summer. There was a significant difference in nitrogen fixation rates among the different termite castes ($F = 24.78$; df = 5, 165; $P < 0.0001$) (Table 1) (Fig. 2). Worker termites had the highest nitrogenase activity of all termite castes (Tukey HSD).

Fresh Mass, Dry Mass, and Body Water. There was a significant difference in fresh mass over the 23 months of the study ($F = 3.52$; df = 22, 667; $P < 0.0001$) (Fig. 3). Fresh masses were greatest in the winter months. There was a significant difference in dry masses over the 23 months of the study with average dry masses highest in the winter months. ($F = 3.77$; df = 22, 667; $P < 0.0001$) (Fig. 4). There was a significant

difference in percent body water over the 23 months of the study (F = 7.7; df = 22, 667; P < 0.0001) (Fig. 5). Percent body water was greatest in summer months.

Caste proportion. There was a significant difference in the caste proportion of termites (F = 1282.9; df = 5, 63; P = <0.001) (Table 1) (Figs 6 and 7) over the study period. Worker termites comprised the highest proportion for each collection period (87%). Immature termite larva comprised the next highest proportion at ≈ 10%. However, there was no seasonal difference in caste proportion among the months of the study (F = 0.00; df = 3, 63; P = 1.0) (Table 1). There was a significant difference in the percent body water among the six termite castes studied (F = 173.07; df = 5, 713; P = <0.0001) (Table 1) (Fig. 8).

Termite Abundance. The population estimates for two *R. virginicus* colonies in logs were 0.49 x 10^6 worker termites for one log measuring 7.4 m in length and 1.78 x 10^6 worker termites for a log measuring 2.7 m in length.

Discussion

Nitrogen fixation has been demonstrated in all termite families (Slaytor and Chappell 1994). Because termites can fix atmospheric nitrogen they may be important to the biogeochemical cycling of N in some ecosystems. Pandey et al (1992) estimated that *Reticulitermes* spp. contribute 125.5 - 445.3 g N • ha^{-1} • y^{-1} in a forest ecosystem. Schaefer and Whitford (1981) estimated *Gnathamitermes tubiformans* (Buckley) contributes 66 g N • ha^{-1} • y^{-1} in a desert ecosystem. However, these estimates were not based on seasonal nitrogen fixation rates.

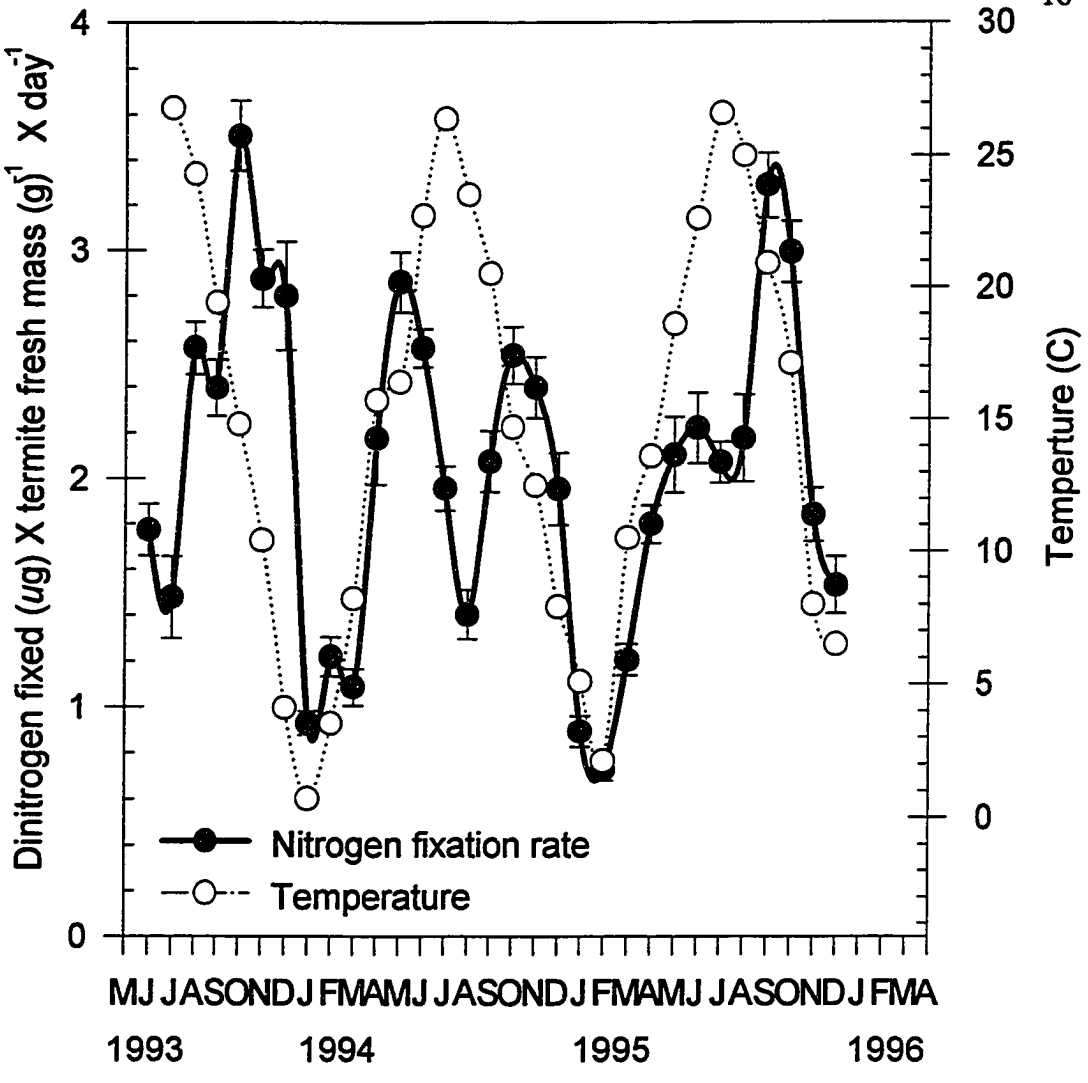

Figure 1. Nitrogen fixation rates (mean and SE) from June 1993 to December 1995. (n=30 for each point of nitrogen fixed)

Table 1. Summary of ANOVA tables for Chapter II.

Monthly Nitrogenase Activity

	df	*F*	*P*
Total	926		
Colony	9	12.37	<0.0001
Month	30	32.92	<0.0001
Error	887		

Caste Nitrogenase Activity

	df	*F*	*P*
Total	165		
Caste	5	24.78	<0.0001
Error	160		

Caste Proportion

	df	*F*	*P*
Total	71		
Season	3	0.00	1.0
Caste	5	1282.19	<0.0001
Error	63		

Percent Body Water of Workers

	df	*F*	*P*
Total	457		
Colony	9	20.85	<0.0001
Month	22	8.09	<0.0001
Error	426		

Percent Body Water of Castes

	df	*F*	*P*
Total	718		
Caste	5	173.07	<0.0001
Error	713		

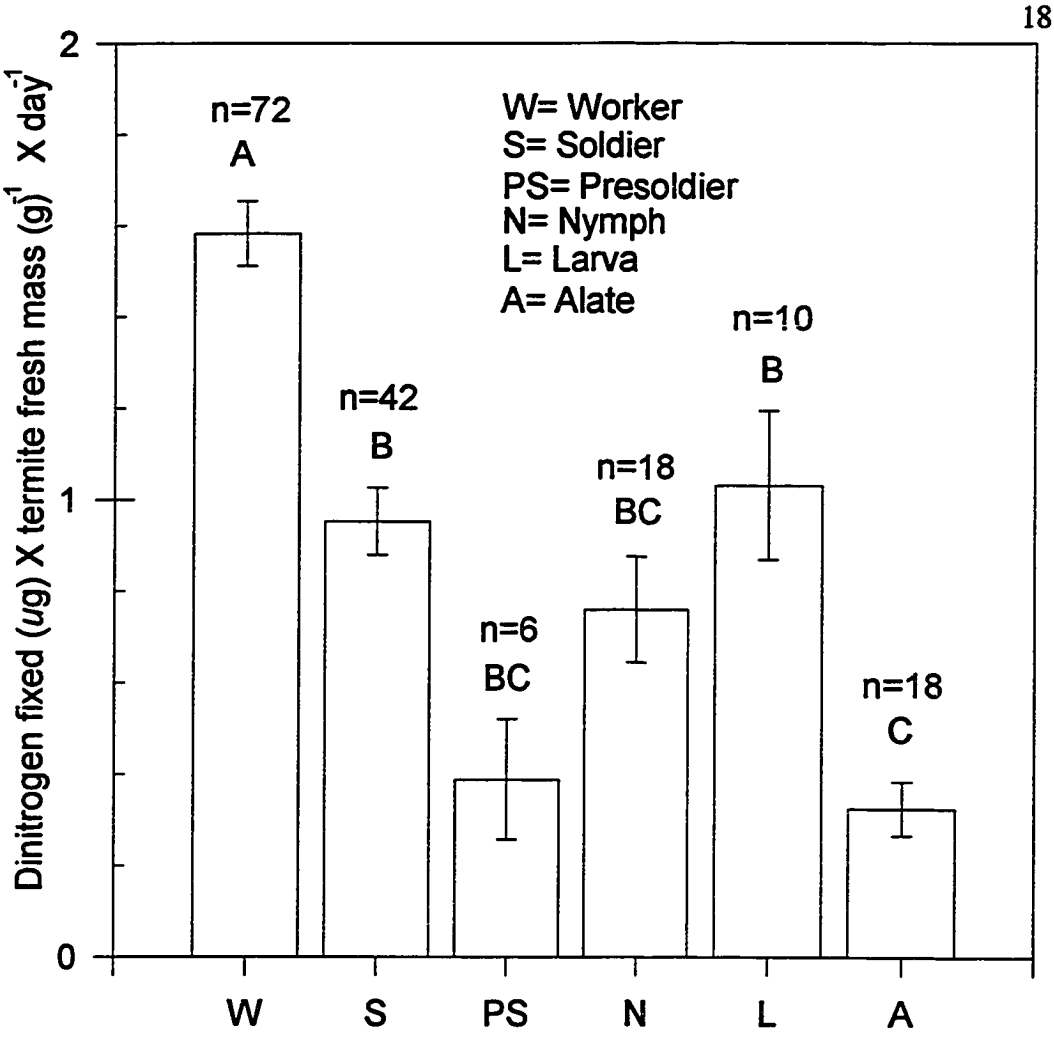

Figure 2. Nitrogen fixation rates (mean and SE) of each termite caste. Bars with the same letters are not significantly different at the 0.05 level.

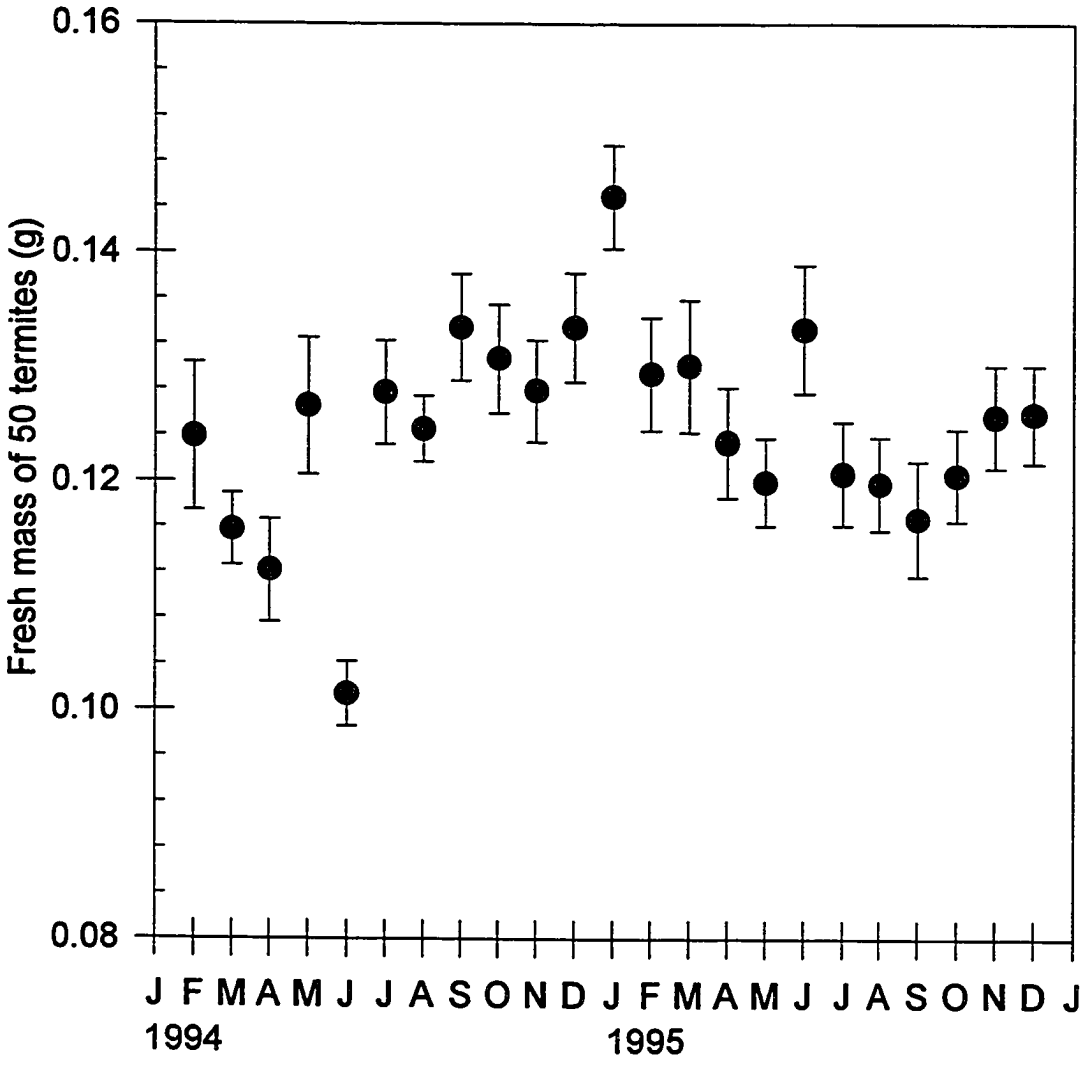

Figure 3. Fresh masses (mean and SE) of termite workers from February 1994 to December 1995.

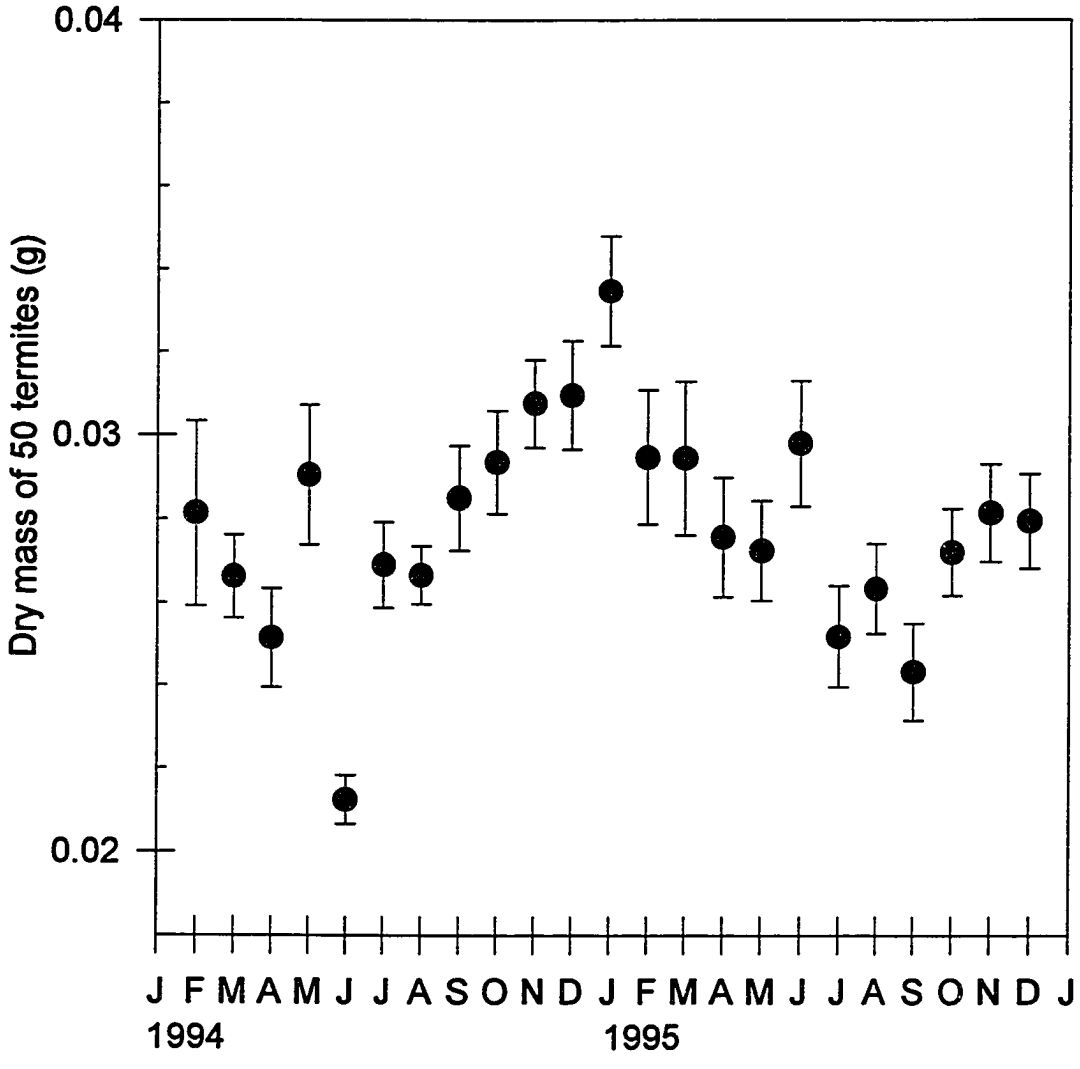

Figure 4. Dry masses (mean and SE) of termite workers from February 1994 to December 1995. (n=30 for each month)

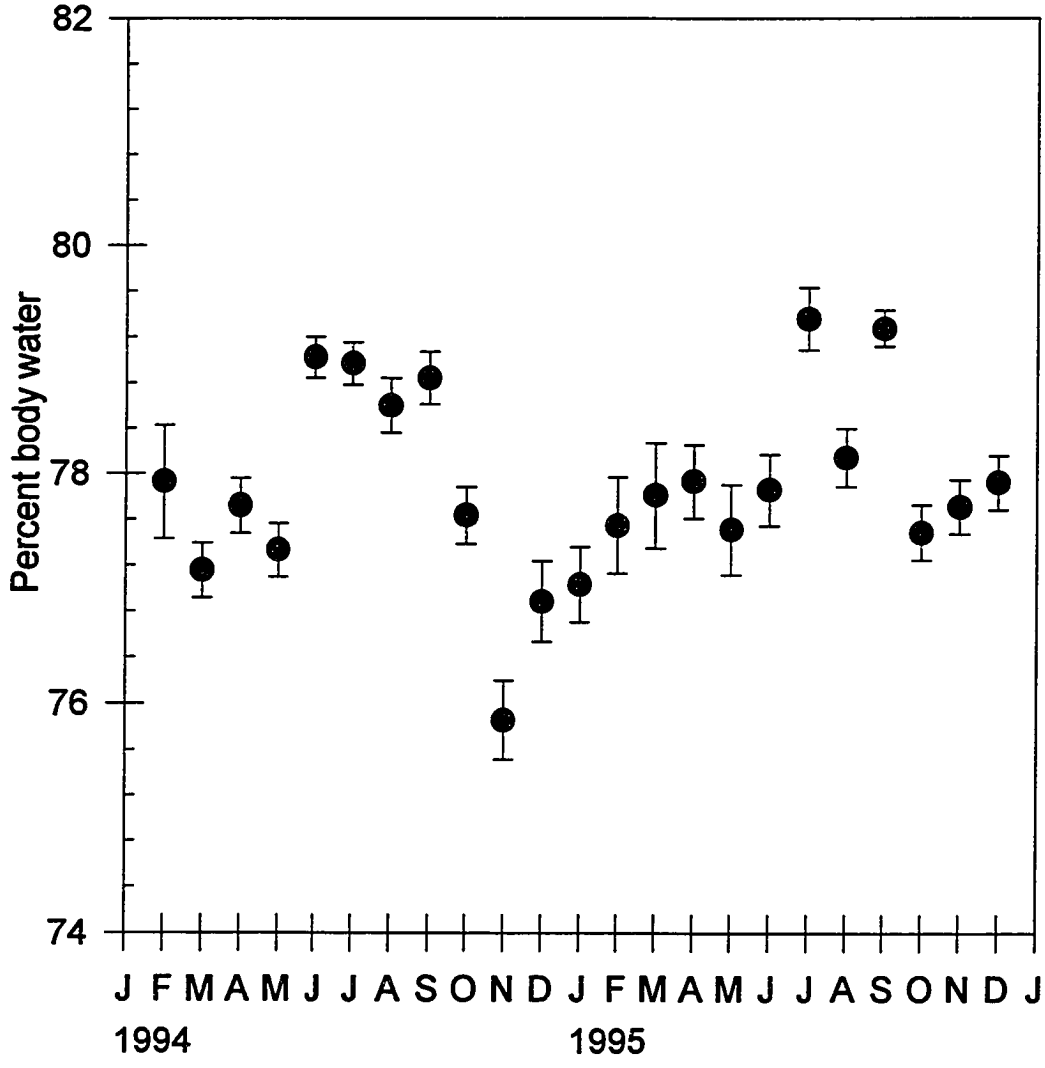

Figure 5. Percent body water (mean and SE) of termite workers from February 1994 to December 1995. (n=30 for each month, each replicate contained 50 termites)

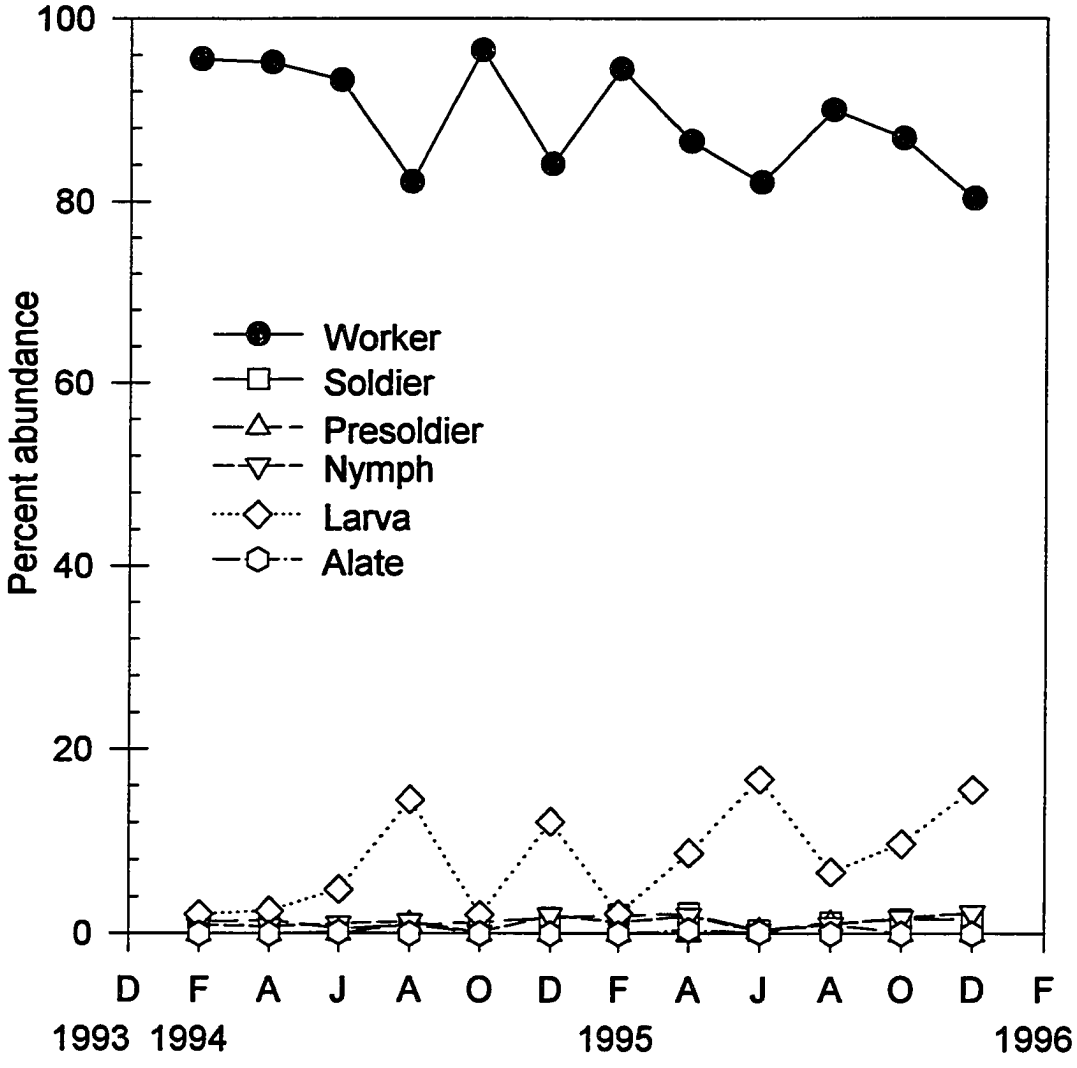

Figure 6. Caste composition from February 1994 to December 1995.

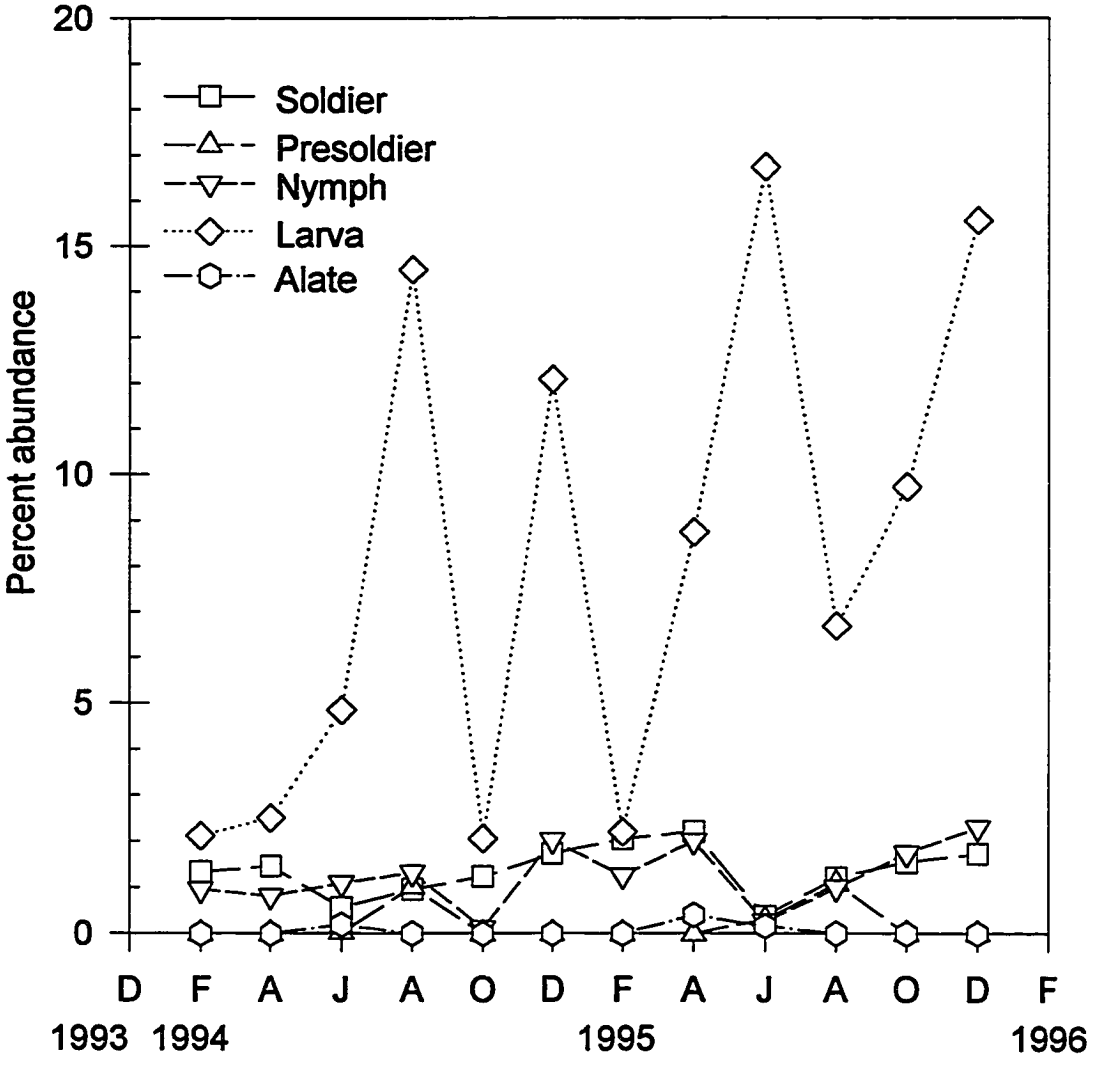

Figure 7. Caste composition from February 1994 to December 1995 without workers.

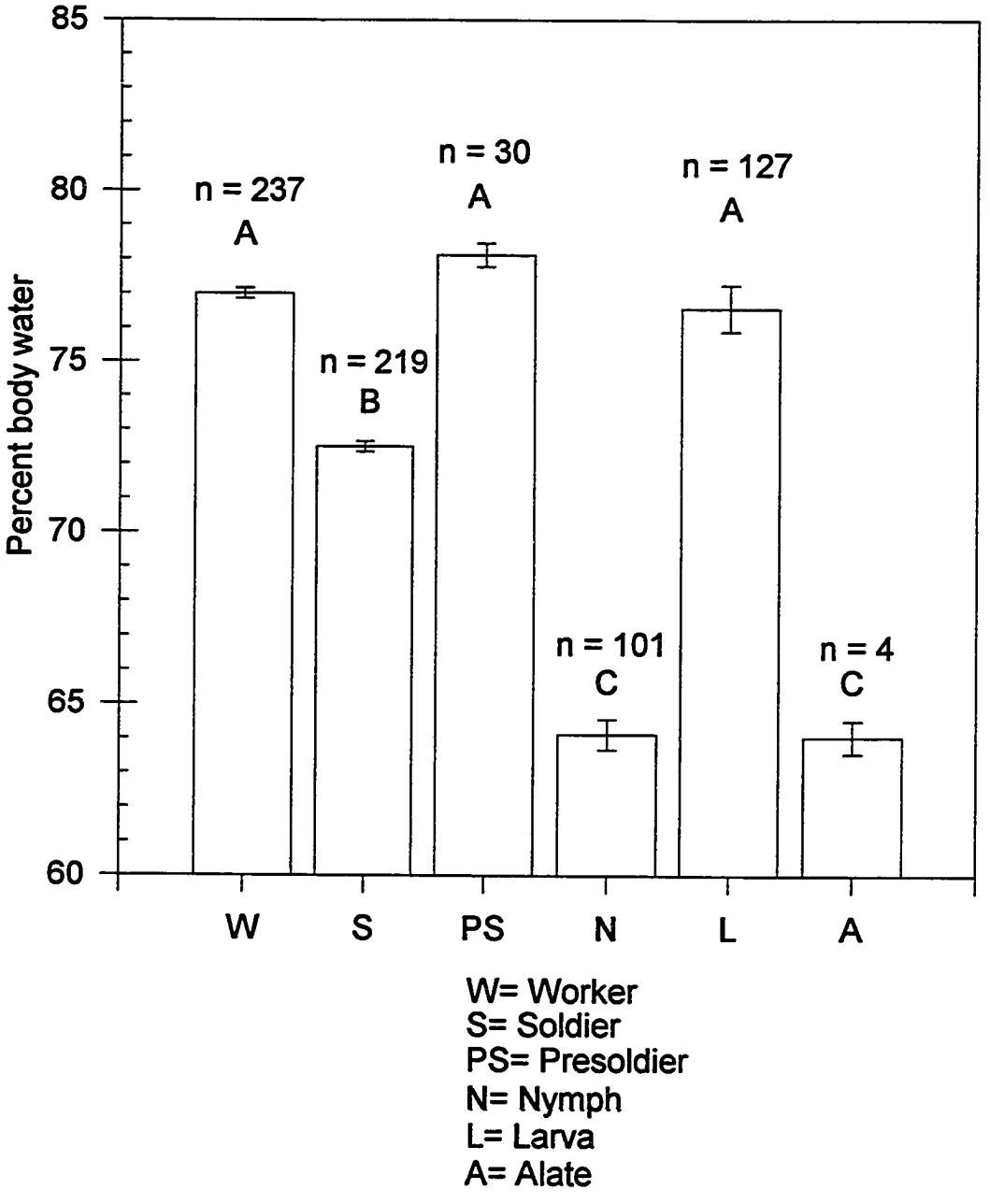

Figure 8. Percent body water of each termite caste. Bars with the same letters are not significantly differrent at the 0.05 level.

Our data indicate that termite nitrogen fixation rates may be related to seasonal temperature changes. Termite nitrogen fixation rates in moderate temperatures (e.g. spring and fall) were nearly double than those during seasonal temperature extremes (e.g. summer and winter). Based on this seasonal data and the abundance data of Howard *et al.* (1982), *Reticulitermes flavipes* and *Reticulitermes virginicus* are capable of contributing 0.94- to 4.68 g N • ha^{-1} • yr^{-1} to forest ecosystems. Using the abundance data from Grace *et al.* (1989), *R. flavipes* and *R. virginicus* are capable of contributing 76.8- to 384.2 g N • ha^{-1} • yr^{-1} to forest ecosystems.

Termites inhabit fallen logs that occur throughout forest ecosystems. Although nitrogen inputs to ecosystems are usually given per hectare, termite nitrogen contributions may be more relevant if viewed as a mosaic of nitrogen "hotspots". We estimated the population size of two *R. virginicus* colonies in logs to have 0.49- and 1.7 x 10^6 worker termites. Based on this abundance data, termites may contribute substantial amounts of nitrogen to logs which may then build up over a season and be released even during low rates of termite nitrogen fixation.

Workers had the highest rates of nitrogenase activity of all castes. Previous studies have indicated that larvae may fix 300 times as much nitrogen as workers in *Coptotermes*, another rhinotermitid (Breznak 1982), but in our study they fixed less than workers. The low rates of nitrogenase activity in non-worker castes coupled with their low numbers in our density samples indicate that their nitrogen contributions to forests is minimal. Higher numbers of these castes might occur in termite nests deep within the soil, but it is unlikely that their nitrogenase activity differs from our measures.

CHAPTER III

VARIATION IN TERMITE NITROGEN FIXATION RATES: RESPONSE TO FIELD AND LABORATORY DIETARY NITROGEN

Introduction

Termites feed primarily on dead plant material (Lee and Wood 1971, Wood 1978, Waller and La Fage 1987a), a food source that has a higher carbon-to-nitrogen ratio than their own tissues (La Fage and Nutting 1978). Although most heterotrophs must consume proteinaceous foods to obtain sufficient nitrogen, termites are able to supplement their low dietary nitrogen by the action of symbiotic nitrogen-fixing bacteria (Bennemann 1973, Breznak *et al.* 1973, French *et al.* 1976). Newly fixed nitrogen is converted into termite tissues (Bentley 1984) and is a nitrogen input to desert (Schaefer and Whitford 1981) and forest ecosystems (Wood and Sands 1978). The ability to fix atmospheric nitrogen has enabled termites to exploit foods low in nitrogen and to achieve an optimal carbon-nitrogen balance (Higashi *et al.* 1992).

Foods high in nitrogen can also be used by termites, although nitrogenase activity in bacterial symbionts may be suppressed under those conditions (Breznak *et al.* 1973, Waughman *et al.* 1981). Termites might select high-nitrogen foods when they are available. For example, Shellman-Reeve (1994) demonstrated a positive correlation between the density of newly flown alates and the total cambium-nitrogen of a log. The nitrogen content of termite foods may be influenced by many factors including fungal

decay (Hudson 1972, Collins 1983). Whether or not termites actually select high nitrogen foods in nature, the nitrogen content of natural foods may influence nitrogen fixation rates. Consequently, the nitrogen contributed to forest ecosystems by termites may vary depending on the nutritional quality of their food.

The objectives of the study were to determine: 1) the nitrogen content of natural foods and relate it to nitrogen fixation rates in termites collected from those food sources and 2) the effect of different sources and concentrations of combined nitrogen on termite nitrogen fixation rates in laboratory experiments.

Materials and Methods

Field Studies. Insects and Study Site. For each collection period, ten colonies of *Reticulitermes* spp. Holmgren were collected at The Nature Conservancy headquarters of the Virginia Coast Reserve located near Nassawadox, in Northampton County, Virginia. Sections of logs infested with termites were returned to the laboratory. Termites removed from the logs were immediately assayed for nitrogenase activity in July 1993, October 1993, January 1994, and April 1994.

Nitrogen Fixation (acetylene reduction assay). The acetylene reduction bioassay was used to determine nitrogen fixation rates for each termite colony. The assay is based on the ability of nitrogenase to reduce acetylene to ethylene at three times the rate that it reduces dinitrogen to ammonia (Hardy et al. 1973, Bently 1984). The following protocol used in this study was adapted from Pandey *et al.* (1992).

Fifty worker termites, removed from their nest material and weighed to the nearest

0.1 mg, were placed in an 8.5-ml glass vial with a rubber sleeve cap septum. One ml of air was removed and 1.0 ml of acetylene was added to the vial, resulting in a final atmosphere of ≈ 12% acetylene. Three replicate vials per colony were incubated at 22 ± 2 C for 30 minutes. Following incubation, a 200-ul sample of head space was removed with a 0.5-ml Hamilton Gas Tight® syringe and injected into a Varian® 3600 gas chromatograph equipped with a flame ionization detector and a Porapak® N column. Ethylene peaks were analyzed using a standard concentration curve with known amounts of ethylene to determine the moles of ethylene produced for each sample. Final nitrogen fixation rates are expressed as dinitrogen fixed (ug)• termite fresh weight $(g)^{-1}$ • day^{-1}.

Nitrogen Content of Wood (TKN method). Wood samples from the termite colonies were cleared of all termites and termite debris and oven-dried at 48 ± 2 C for ≈ 48 hours. Three replicate wood samples for each colony were ground with a Wiley® mill equipped with a 20-mesh screen (850 μm aperture size) after which total Kjeldahl nitrogen (TKN) was determined for each sample using a Carlo Erba CHN analyzer.

Statistical Analyses. A completely randomized ANOVA was used to detect differences in nitrogen content among the wood collected for each colony within a collection period and also among collection periods. Multiple regression analysis was used to determine if the nitrogen content of the wood affected termite nitrogen fixation rates, adjusting for temperature (SAS Institute, 1990).

Laboratory Studies. Insects and Study Site. Termites from *Reticulitermes* spp. colonies were collected from southeastern Virginia from June, 1994 through December, 1994. The termites used for the ammonium phosphate and uric acid experiments were

collected in July, 1995. Five colonies were used to determine the effects of dietary nitrogen on termite nitrogen fixation rates in each of six laboratory experiments.

Nitrogen Diet. Sources of nitrogen used in the feeding experiments were ammonium nitrate, ammonium phosphate, amino acid mixtures, urea, and uric acid. Two experiments were conducted with a mixture of equal amounts of three amino acids. In the first amino acid experiment, a mixture of histidine, serine, and aspartic acid was used; in the second, a mixture of proline, tryptophan, and leucine was used.

One- and 3% solutions were prepared for each nitrogen source. The nitrogen content of the filter paper was calculated based on a w/w basis later. In each experiment, 1.0 ml of the solutions was applied to a 9.0-cm diameter Whatman® filter paper; a control treatment was prepared by adding 1.0 ml of deionized water to the filter paper. The wet treated paper was then placed into a 1-pint plastic container that had been randomly assigned to each treatment. Each plastic container was first prepared by adding approximately 70 ml of vermiculite and 35 ml of deionized water; 100 termites were added to the container after the water had been absorbed by the vermiculite. Each container was covered with a vented plastic lid. The containers were then placed into an incubator at 26 $\pm$ 2 C. Termites from five colonies were used for each of the six experiments. There were three replicate containers for each treatment with 100 termites per replicate for each colony.

Three weeks later, 50 termites were removed from each container and assayed for nitrogenase activity. Containers having fewer than 20 termites were not assayed for nitrogenase activity and were reported as missing data points in the statistical analysis.

Statistical Analysis. A randomized complete block design ANOVA was used to partition colony variation for each experiment. The Tukey honestly significant difference (HSD) test was used to compare means among the treatments (SAS Institute, 1990).

Results

Field Studies. There were significant differences in the nitrogen content of natural termite foods within each collection period: July 1993 ($F = 32.28$, df = 9,20, $P < 0.001$), October 1993 ($F = 39.09$, df = 9,20, $P < 0.001$), January 1994 ($F = 139.97$, df = 9,20, $P < 0.001$), and April 1994 ($F = 101.04$, df = 9,20, $P < 0.001$) (Fig. 9). Furthermore, there was a significant difference in the nitrogen content among the collection periods ($F = 49.87$, df = 3,116, $P < 0.001$) (Fig. 10). However, there was no relationship between the nitrogen content of the wood and termite nitrogen fixation rates within a collection period or among collection periods (Fig. 11).

Laboratory Studies. There was a significant difference in nitrogen fixation rates in termites maintained on a diet enriched with ammonium nitrate ($F = 6.05$, df = 2,31, $P = 0.0048$) (Fig. 12). Termites fed a 3% solution (5% w/w) of ammonium nitrate had significantly lower nitrogen fixation rates than termites fed on either 1% solution of (2% w/w) ammonium nitrate or water-treated filter paper. The nitrogenase activity of termites fed a 1 and 3% solution of ammonium phosphate (2% and 5% w/w, respectively) did not differ from water-treated controls (Tukey HSD) (Fig. 12). A 3% solution (5% w/w) of a mixture of the amino acids proline, tryptophan, and leucine significantly reduced termite nitrogenase activity compared with water-treated controls ($F = 5.39$, df = 2,38, $P =$

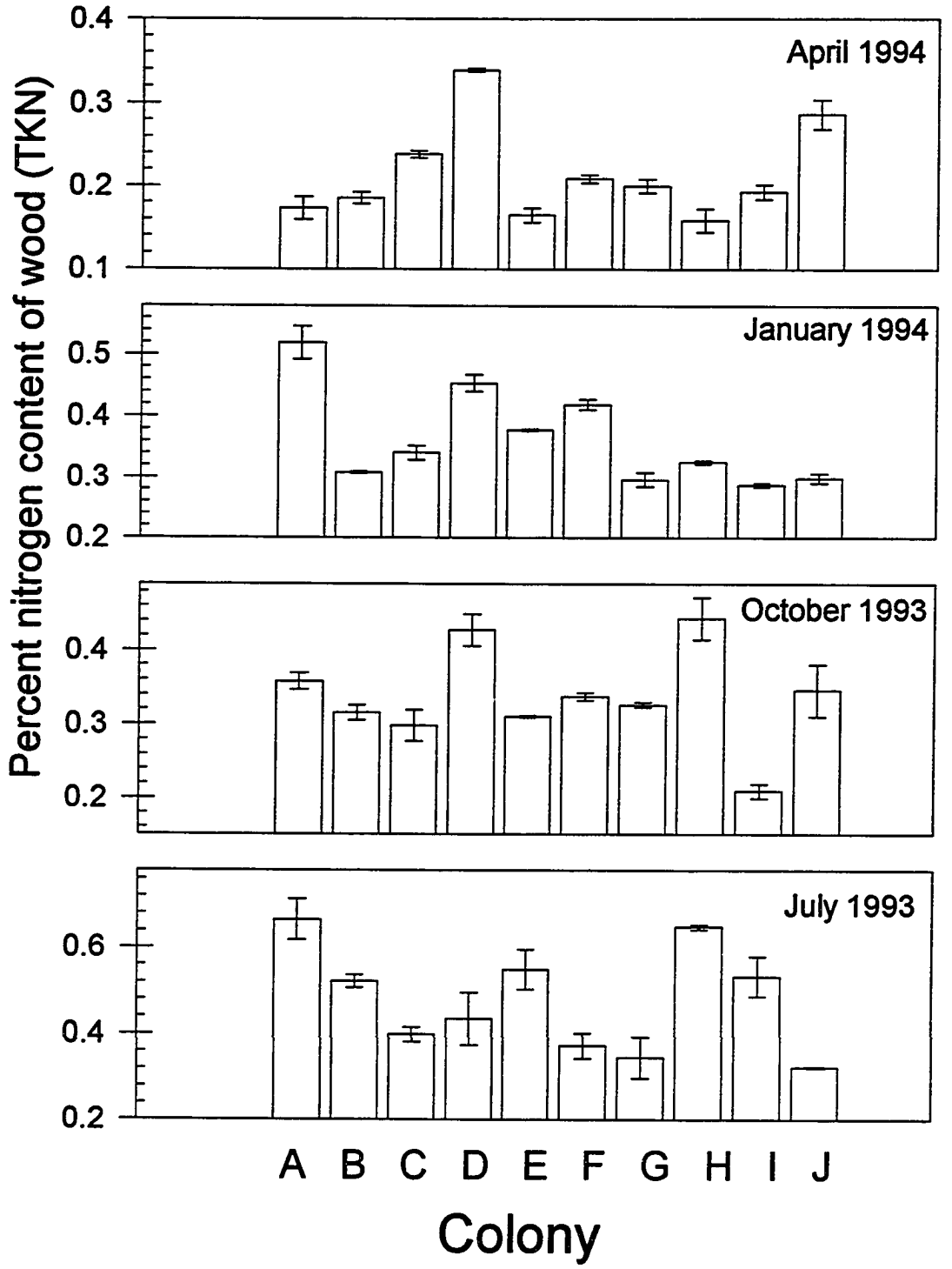

Figure 9. Total Kjeldahl nitrogen (TKN) (mean and SE) of wood collected from 10 *Reticulitermes* colonies (A through J) for July 1993, October 1993, January 1994, and April 1994. (n = 3 for each colony)

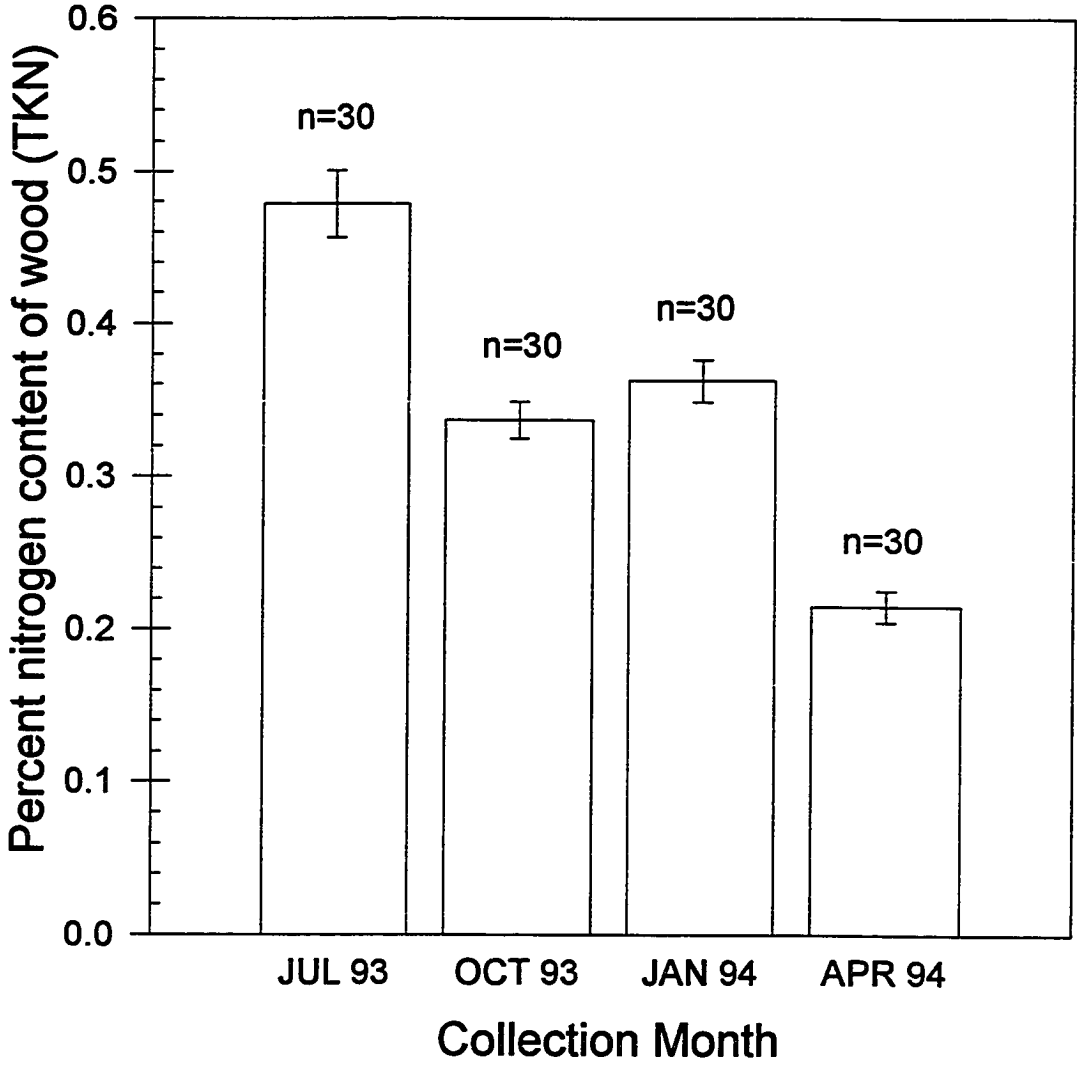

Figure 10. Total Kjeldahl nitrogen (TKN) (mean and SE) of wood collected from 10 *Reticulitermes* colonies (A through J) per collection month.

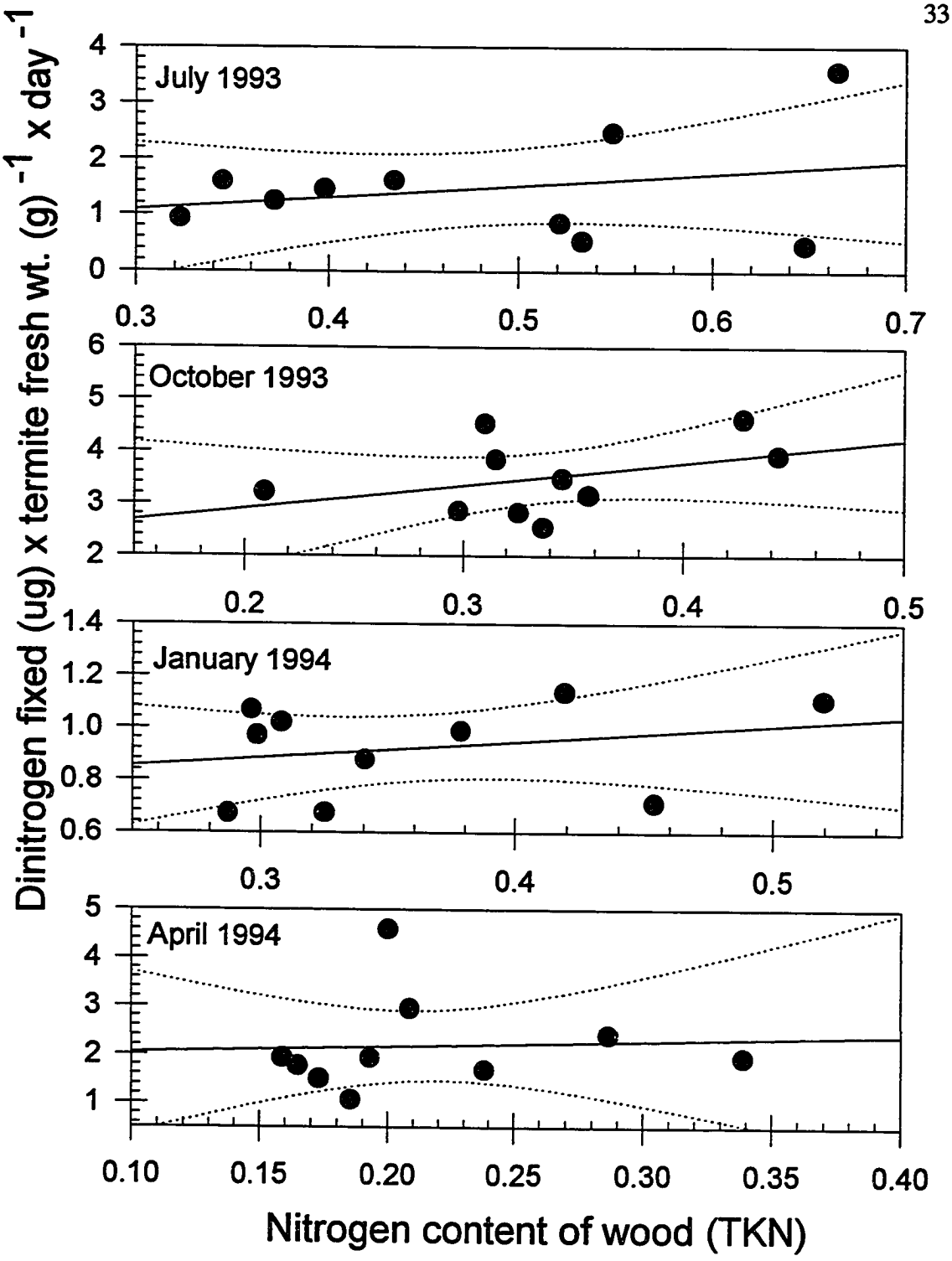

Figure 11. Termite nitrogen fixation rates vs. nitrogen content of wood. Each point represents a different colony and is the mean of three replicates for nitrogen fixation rates and wood nitrogen content.

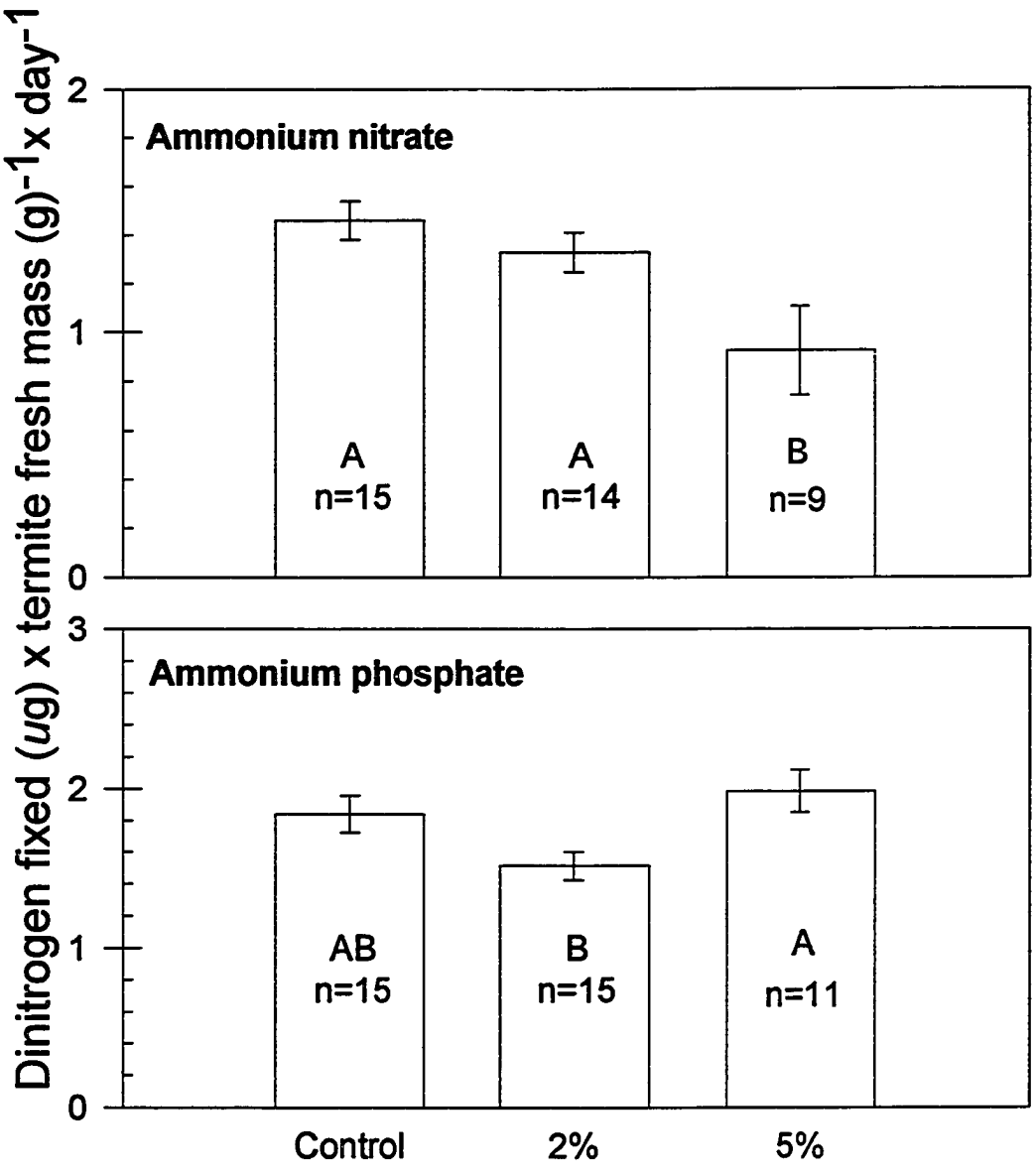

Figure 12. Termite nitrogenase activity (mean and SE) in response to increased dietary nitrogen from ammonium nitrate and ammonium phosphate. Bars with the same letter are not different at the 0.05 level.

0.0087) (Fig. 13). Nitrogen fixation rates for termites maintained on a mixture of the amino acids histidine, serine, and aspartic acid tended to be lower than controls, but no significant difference was detected ($F = 0.59$, df = 2,38, $P = 0.5598$) (Fig. 13). The nitrogen fixation rates of termites fed 1% and 3% solutions (2% and 5% w/w, respectively) of urea did not differ from water-treated controls ($F = 0.15$, df = 2,38, $P = 0.8602$) (Fig. 14). Termites fed a 1% solution (2% w/w) of uric acid had significantly higher nitrogen fixation rates than termites fed the water-treated control ($F = 4.51$, df = 2,37, $P < 0.0177$) (Fig. 14).

Discussion

The nitrogen content of natural termite food is a function of physical deterioration and microbial action (La Fage and Nutting 1978, Martin 1979). The nitrogen content of wood may range from 0.03 to 0.1% for heartwood (La Fage and Nutting 1978, Collins 1983) to 0.5% for the cambium layer (Haack and Slansky 1987). The nitrogen content of the wood in our experiment falls within this range. However, there was no relationship between nitrogenase activity and the nitrogen content of natural termite foods either within each collection period or over all collections. These results suggest that the amount of nitrogen contributed to forest ecosystems by termites is not affected by nitrogen availability within the range of natural wood-nitrogen.

Previous studies showed that artificial diets higher in nitrogen than natural termite foods suppress termite nitrogenase activity (Breznak *et al.* 1973, Waughman *et al.* 1981). Our laboratory studies indicated that the source of nitrogen enrichment, as well as the

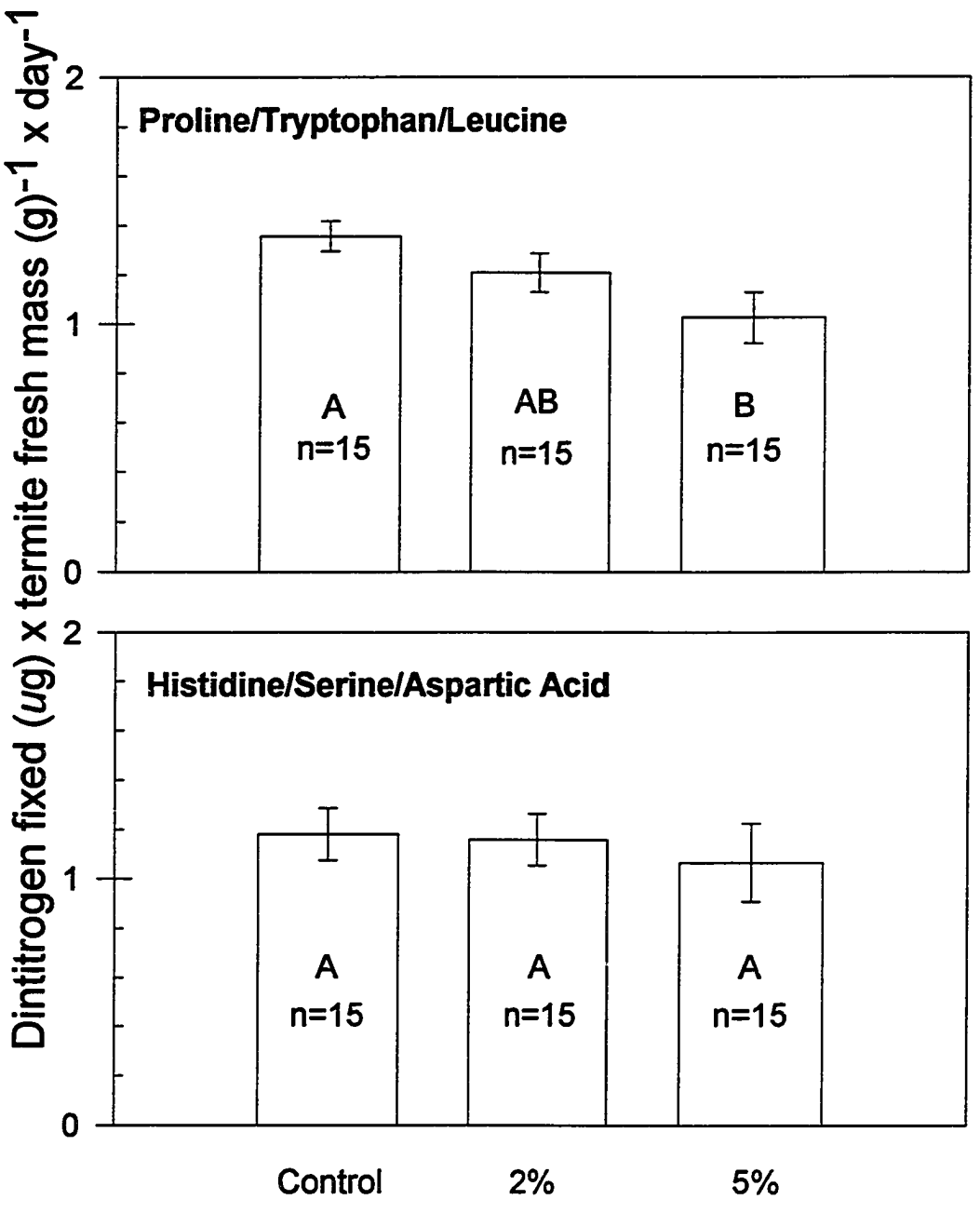

Figure 13. Termite nitrogenase activity (mean and SE) in response to increased dieatry nitrogen from a mixture of the amino acids: proline, tryptophan, leucine and from a mixture of histidine, serine, aspartic acid. Bars with same letter are not significantly different at the 0.05 level.

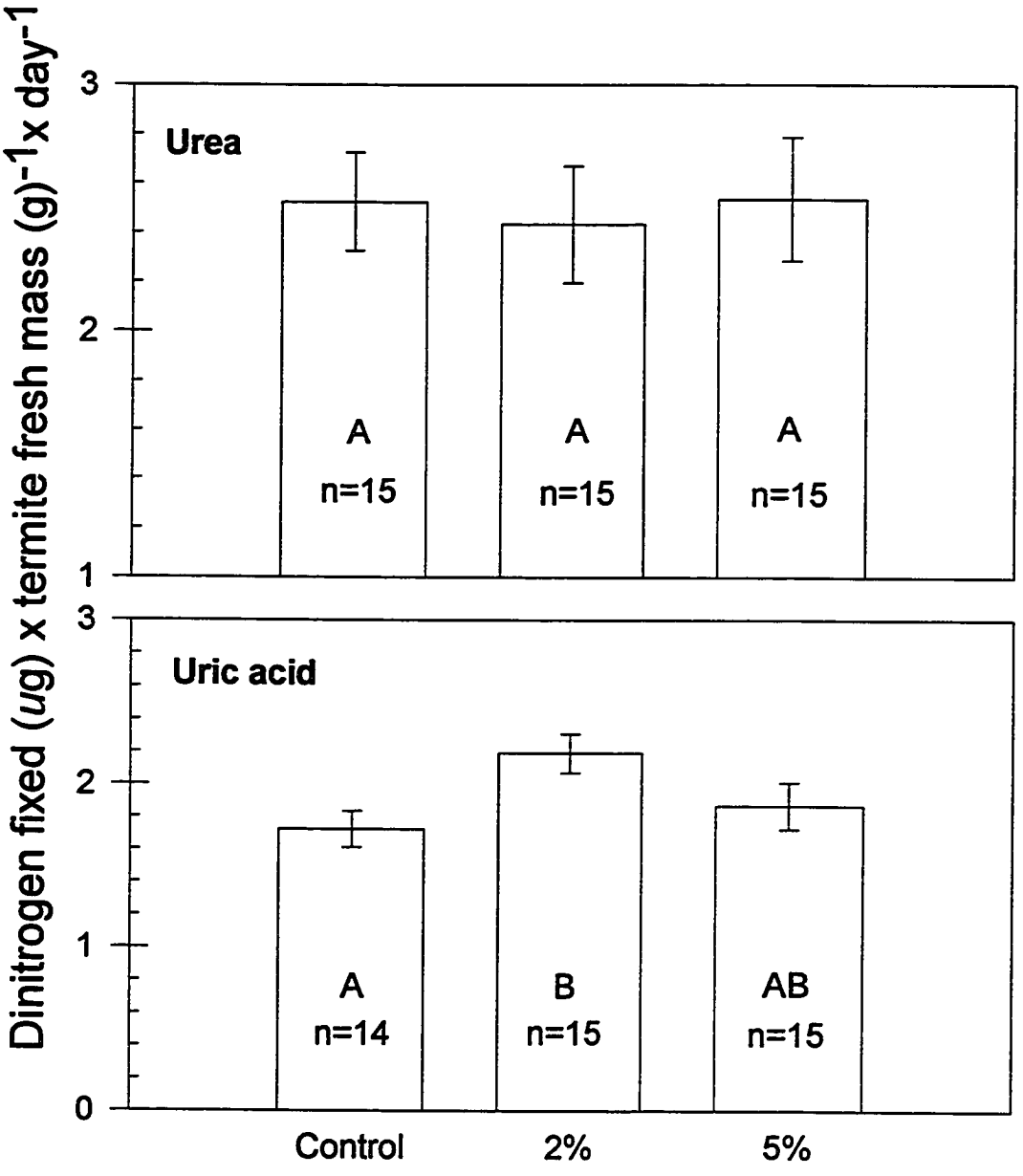

Figure 14. Termite nitrogenase activity (mean and SE) in response to increased dietary nitrogen from urea and uric acid. Bars with the same letter are not significantly different at the 0.05 level.

concentration of nitrogen, were important factors affecting termite nitrogen fixation rates. Termites that were maintained on a diet of filter paper treated with 1 and 3% solutions (2% and 5% w/w, respectively) of ammonium nitrate had significantly less nitrogenase activity than termites fed water-treated controls. Symbiotic nitrogen fixation in plants shows the same trend. Nitrogen fixation rates of *Trifolium repens* Blanca (white clover) decreases when plants are supplied with NO_3^- (Macduff *et al.* 1989). Butler (1988) showed a similar decrease in nitrogen fixation rates in *Medicago littoralis* Harbinger (medic) in response to nitrate. However, nitrogen fixation rates in *Trifolium vesiculosum* Savi (arrowleaf clover) depend on the concentration of NO_3^- applied and the soil type (Schomberg and Weaver 1990).

Decreased nitrogenase activity may be linked to the NH_4^+ portion of ammonium nitrate solution because of its role in the suppression of nitrogenase transcription (Gottschalk 1986). Ammonia suppresses nitrogenase activity in the free living bacterium *Klebsiella pneumoniae* (Gussin *et al.* 1986). Chappell and Slaytor (1986) attribute the control of nitrogenase activity in the symbiotic bacteria of *Nasutitermes walkeri* Hill to fluxes in NH_4^+ concentration. Breznak *et al.* (1973) suppressed termite nitrogenase activity with dietary supplements of ammonium salts. Termites can encounter NH_3 from the excretions of terrestrial isopods and some cockroach species (Slaytor and Chappell 1994) with which they share nest material (Curtis and Waller unpublished data). Although there was a decrease in termite nitrogen fixation rates in response to ammonium nitrate, termites fed 1- and 3% solutions (2%-and 5% w/w, respectively) of ammonium phosphate did not differ from water-treated controls.

In the amino acid experiments, termite nitrogen fixation rates decreased for one of the mixtures and not for the other, although the trend was similar. However, the relationship between amino acid nitrogen sources and termite nitrogen fixation rates remains unclear. Amino acids may be sources of NH_3 which can repress nitrogenase synthesis (Postgate 1982). However, Shanmugam and Morandi (1976) indicate glutamine as regulating nitrogenase independent of NH_3.

The urea and uric acid experiments also showed an inconclusive effect of increased dietary nitrogen on termite nitrogen fixation rates. The 1% and 3% solutions (2% and 5% w/w, respectively) of urea had no effect on termite nitrogen fixation rates. However, a 1% solution (2% w/w colloidal suspension) of uric acid increased termite nitrogenase activity, but the 3% urea solution (5% w/w colloidal suspension) did not differ from water-treated controls. This is further evidence that termite nitrogen fixation rates depend on more than just the quantity of nitrogen in their diet.

Termites accumulate uric acid in the fat body when maintained in the laboratory (Lovelock *et al.* 1985, Potrikus and Breznak 1980a, Chappell and Slaytor 1991). However, uricase is not present in termites to mobilize the stored nitrogen (Potikus and Breznak 1980a, Potrikus and Breznak 1981). Some termites can mobilize the nitrogen in uric acid by association with anaerobic uricolytic bacteria (Potrikus and Breznak 1977, Potrikus and Breznak 1980b). If these bacteria are not symbiotic, then termites must ingest them with their food to maintain an effective population within the gut. The termites in the present study were isolated from their nest material and maintained for three weeks in the laboratory prior to the assay for nitrogenase activity. Therefore, a

change in gut flora related to laboratory confinement may have influenced uric acid metabolism.

In conclusion, our field studies showed that there are differences in the amount of nitrogen in natural termite foods. However, this variation was not correlated with termite nitrogen fixation rates. The nitrogen concentrations used in our laboratory studies were much higher than those we measured in wood eaten by termites in nature, but the effect on termite nitrogen fixation rates was minimal. In the laboratory, termite nitrogen fixation rates were dependent on the source and concentration of dietary nitrogen. It is unclear whether nitrogen concentrations in natural foods ever reach levels high enough to influence nitrogenase activity in termites. Full understanding of the relationship between dietary nitrogen and termite nitrogen fixation rates will require further investigation.

CHAPTER IV

THE EFFECTS OF DECREASED pO_2 AND INCREASED pCO_2 ON NITROGEN FIXATION RATES IN TERMITES[2]

Introduction

Nitrogen fixation has been demonstrated in many species of termites (Breznak, 1982). The ability to fix atmospheric nitrogen makes some termites particularly important to the biogeochemical cycling of nitrogen in desert (Schaefer and Whitford 1981) and forest ecosystems (Wood and Sands 1978). Factors affecting termite nitrogen fixation rates include food quality (Breznak *et al.* 1973, Prestwich *et al.* 1980), termite caste (Breznak *et al.* 1973, Hewitt *et al.* 1987), and termite size (Waller *et al.* 1989).

Decreased pO_2 and increased pCO_2 are likely to occur deep within termite galleries, and these conditions may affect termite nitrogen fixation rates. Subterranean termites construct elaborate galleries within logs that may extend 8.5 m below the surface of the soil (Watson 1960). Paim and Beckel (1964) reported pO_2 = 0.05 atm within decayed logs, and pCO_2 = 0.052 atm has been measured in nests of fungus growing termites of the Macrotermitinae (Matsumoto 1977).

Symbiotic and asymbiotic nitrogen fixation is inhibited by oxygen (Burris *et al.* 1955, Parker and Scutt 1960, Bergersen 1962). However, the effect of decreased pO_2 on termite nitrogen fixation has not been determined. Elevated pCO_2 has various

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consequences for different insect species (reviewed in Nicolas and Sillans 1989), but its role in nitrogen fixation in termites has not been investigated. If O_2 and CO_2 concentrations affect nitrogen fixation rates in termites, then their influence must be considered in estimates of nitrogen contributions by termites to ecosystems.

The objectives of the study were to determine the effects of decreased pO_2 and increased pCO_2 on nitrogen fixation rates in the subterranean termites *Reticulitermes flavipes* (Kollar) and *Reticulitermes virginicus* (Banks).

Materials and Methods

Insects and Study Site. Colonies of *R. flavipes* and *R. virginicus* were collected from pine and mixed hard-wood forests in southeastern Virginia from August, 1994, through October, 1994. Both species are common in this region, but they were not identified to species because no alates were present. Voucher specimens from each colony have been deposited in the reference collection at Old Dominion University.

Nitrogen Fixation (acetylene reduction assay). The acetylene reduction assay was used to determine nitrogenase activity. The assay is based on the ability of nitrogenase to reduce acetylene (C_2H_2) to ethylene (C_2H_4) at three times the rate at which dinitrogen (N_2) is reduced to ammonia (NH_3) (Hardy *et al.* 1973, Bentley 1984).

In all experiments, the vials were incubated at 22 $\pm$ 2 C for 30 minutes (Pandey *et al.* 1992). Following incubation, a 200 ul sample of head space was removed from each vial with a 0.5 ml Hamilton gas tight syringe (Hamilton Company, Reno, Nevada) and injected into a Varian 3600 gas chromatograph (Varian Instrument Group, Walnut

Creek, California) equipped with a flame ionization detector and a Porapak N column (Alltech, Deerfield Illinois). Ethylene peaks were analyzed using a standard concentration curve with known amounts of ethylene to determine the moles of ethylene produced for each sample. For each colony, one replicate vial with termites, without acetylene added, was incubated with the other treatment vials and examined for spontaneous ethylene production. Final rates are expressed as N_2-fixed (ug) • termite fresh mass $(g)^{-1}$ • day^{-1}.

For all of the following experiments, the original air in the vials was assumed to have the following composition: $pN_2 = 0.780$ and $pO_2 = 0.2099$ (Kotz and Purcell 1991). The volume of termites in the vials was 0.17 ml and considered negligible in the gas composition calculations. Termite oxygen consumption during the 30 minute acetylene reduction assay was also considered negligible (Odelson and Breznak 1983).

Experiment I. Decreased pO_2, increased pCO_2. To test the effects of decreased pO_2 and increased pCO_2 on termite nitrogen fixation, we designed the following experiment. Nine replicate units (8.5 ml vials) were prepared for each of five termite colonies. Each unit was randomly assigned one of three treatments: control, $pCO_2 = 0.010$, and $pCO_2 = 0.100$. The control experimental units were prepared by weighing 50 worker termites to the nearest 0.1 mg and placing them into an 8.5 ml glass vial with a rubber sleeve-cap septum. One milliliter of air was removed, and then 1.0 ml of acetylene was injected into each vial in the control treatment. This resulted in an atmosphere containing $pC_2H_2 = 0.116$ and $pO_2 = 0.186$.

Atmospheres of $pCO_2 = 0.010$ and $pCO_2 = 0.100$ were generated in glass vials by removing 0.1 or 1.0 ml of air in the vials and injecting 0.1 or 1.0 ml with CO_2,

respectively. Next, 1.0 ml of the gas in the vial was removed and 1.0 ml of acetylene was injected. After the replacement of 1.0 ml of air with 1.0 ml of acetylene, the gas composition within the units was $pC_2H_2 \approx 0.115$ and $pCO_2 = 0.010$ or $pCO_2 = 0.100$. The pO_2 of the control, $pCO_2 = 0.010$ and $pCO_2 = 0.100$ treatments was 0.186, 0.183, and 0.162, respectively. The vials were assayed using the acetylene reduction assay described above. Gas composition calculations are based on $pO_2 = 0.210$ for breathing air, 22 ^{0}C, and 8.5 ml vials.

Experiment II. Decreased pO_2, increased N_2 and argon. This experiment was designed to determine whether decreased pO_2 had an effect on nitrogen fixation rates independent of the effects of elevated pCO_2. Fifty worker termites were removed from their nest material, weighed to the nearest 0.1 mg, and placed in an 8.5 ml glass vial with a rubber sleeve-cap septum; nine units were prepared for each of five termite colonies. Each unit was randomly assigned to one of three treatments: control, N_2, or argon.

The control group was prepared as described earlier and had an atmosphere of $pC_2H_2 = 0.116$, $pO_2 = 0.186$, and $pN_2 \approx 0.70$. The N_2 and argon treatments were prepared similarly except that prior to the replacement of 1.0 ml of air with acetylene, 1.0 ml of air was replaced with N_2 or argon respectively. This resulted in an atmosphere of $pC_2H_2 \approx 0.115$, $pO_2 = 0.164$, and the remainder N_2 ($pN_2 = 0.713$) or N_2 ($pN_2 = 0.610$) and argon ($pAr = 0.103$) with less than ambient levels of CO_2. Samples were assayed using a gas chromatograph as described above.

Experiment III. Constant pO_2, increased pCO_2. We designed this experiment to examine the effects of increased pCO_2 while maintaining constant pO_2 in the treatment

vials. Six replicate units were prepared for each of five termite colonies. Each vial was randomly assigned one of the following treatments: control or $pCO_2 = 0.098$. The vials in the control treatment group were vented with a needle and flushed for 30 seconds with N_2 at a flow rate of 7.12 ml/second. This was enough time to evacuate the vial, leaving approximately 1.46×10^{-92} ml of air originally in the vial. Next, 1.8 ml of the gas in the vial was removed and 1.8 ml of O_2 was injected into the vial. Then 1.0 ml of vial gas was removed and 1.0 ml of acetylene was injected. Each unit in the control treatment had an atmosphere of $pC_2H_2 = 0.113$, $pO_2 \approx 0.18$, and $pN_2 = 0.706$.

The $pCO_2 = 0.098$ treatment group was similarly flushed for 30 seconds with N_2 at a flow rate of 7.12 ml/second. Therefore, these vials were also flushed of all the original air. Next, 1.2 ml of the gas in the vial was removed and 1.2 ml of CO_2 was injected. Then 1.8 ml of the gas mixture in the vial was removed and 1.8 ml of O_2 was injected. Next, 1.0 ml of the vial gas was removed and 1.0 ml of acetylene was injected into the vial. The final gas composition in the $pCO_2 = 0.098$ treatment group was $pC_2H_2 = 0.111$, $pO_2 \approx 0.18$, $pCO_2 = 0.098$, and $pN_2 = 0.611$.

Experiment IV. 5.2% & 10.3% O_2, N_2, or CO_2 flush. Experiment IV was designed to test the effects of very low pO_2 on termite nitrogen fixation rates. Here, vials were flushed out with N_2 or CO_2 for 30 seconds at a flow rate of 7.12 ml/second before replacement with the appropriate amount of O_2. The amount of original gas remaining in the vials after flushing was approximately 1.46×10^{-92} ml and was considered negligible in the gas composition calculations.

Fifty worker termites were weighed to the nearest 0.1 mg and placed into an 8.5

ml vial. Fifteen units were prepared for each of five termite colonies. Each experimental unit was randomly assigned to one of five treatments: control, $pO_2 = 0.052$ (N_2 flush), $pO_2 = 0.103$ (N_2 flush), $pO_2 = 0.052$ (CO_2 flush), or $pO_2 = 0.103$ (CO_2 flush).

The control vials were prepared as described earlier for Experiments I & II, and had a $pO_2 = 0.186$ and $pC_2H_2 = 0.116$. The $pO_2 = 0.103$ treatment vials were vented with a needle and flushed with N_2 or CO_2 for 30 s at a flow rate of 7.12 ml/sec; 1.0 ml of gas was then removed and 1.0 ml of O_2 was injected into the vial. Next, another 1.0 ml of gas was removed and 1.0 ml of acetylene was injected into the vial. This resulted in a final atmosphere of $pO_2 = 0.103$, $pC_2H_2 = 0.115$, and $pN_2 = 0.782$ (for N_2 flushed vials), or $pCO_2 = 0.782$ (for CO_2 flushed vials).

The $pO_2 = 0.052$ treatment vials were prepared similarly except that after flushing the vials with either N_2 or CO_2, 0.5 ml of gas was removed and 0.5 ml of O_2 was injected into the vial. Then 1.0 ml of gas was removed and 1.0 ml of acetylene was injected. This resulted in a final atmosphere of $pO_2 = 0.052$, $pC_2H_2 = 0.116$, and $pN_2 = 0.832$ (for N_2 flushed vials), or $pCO_2 = 0.832$ (for CO_2 flushed vials).

Statistical Analysis. For each experiment, a randomized complete block design analysis of variance (ANOVA) was used to partition colony effects and to test for a difference among treatment means. The Tukey honestly significant difference (HSD) multiple comparison test was used to compare means in experiments with three or more treatments (SAS Institute, 1990).

Results

Experiment I. Decreased pO_2, increased pCO_2. The nitrogen fixation rates for termites exposed to an atmosphere of $pO_2 = 0.162$ and $pCO_2 = 0.100$ were significantly higher than the nitrogen fixation rates of controls at $pO_2 = 0.186$ and ambient CO_2 ($F = 4.24$; df = 2, 38; $P = 0.0217$) (Table 2) (Fig. 15)

Experiment II. Decreased pO_2, increased nitrogen and argon. The nitrogen fixation rates for termites exposed to an atmosphere of $pO_2 = 0.164$ generated by the addition of argon were significantly higher than those of controls with an atmosphere of $pO_2 = 0.186$ ($F = 6.07$; df = 2, 38; $P = 0.0052$) (Table 2) (Fig. 16). Nitrogen fixation rates of termites in increased pN_2 atmospheres tended to be higher than controls but the difference was not significantly different (Table 2) (Fig. 16).

Experiment III. Constant pO_2, increased pCO_2. The nitrogen fixation rates for termites exposed to $pCO_2 = 0.098$ ($pO_2 \approx 0.18$) did not differ from controls at ambient CO_2 and $pO_2 \approx 0.18$ ($F = 0.72$; df = 1, 24; $P = 0.4061$) (Table 2) (Fig. 17).

Experiment IV. 5.2% & 10.3% pO_2, N_2 or CO_2 flush. The nitrogen fixation rates for termites exposed to atmospheres of $pO_2 = 0.052$ and $pO_2 = 0.103$ (remainder N_2) were higher than controls with $pO_2 = 0.186$. Termites in N_2 flushed vials also had higher nitrogen fixation rates than termites in CO_2 flushed vials, probably because the latter were immobilized due to narcosis ($F = 48.6$; df = 4, 66; $P < 0.0001$) (Table 2) (Fig. 18).

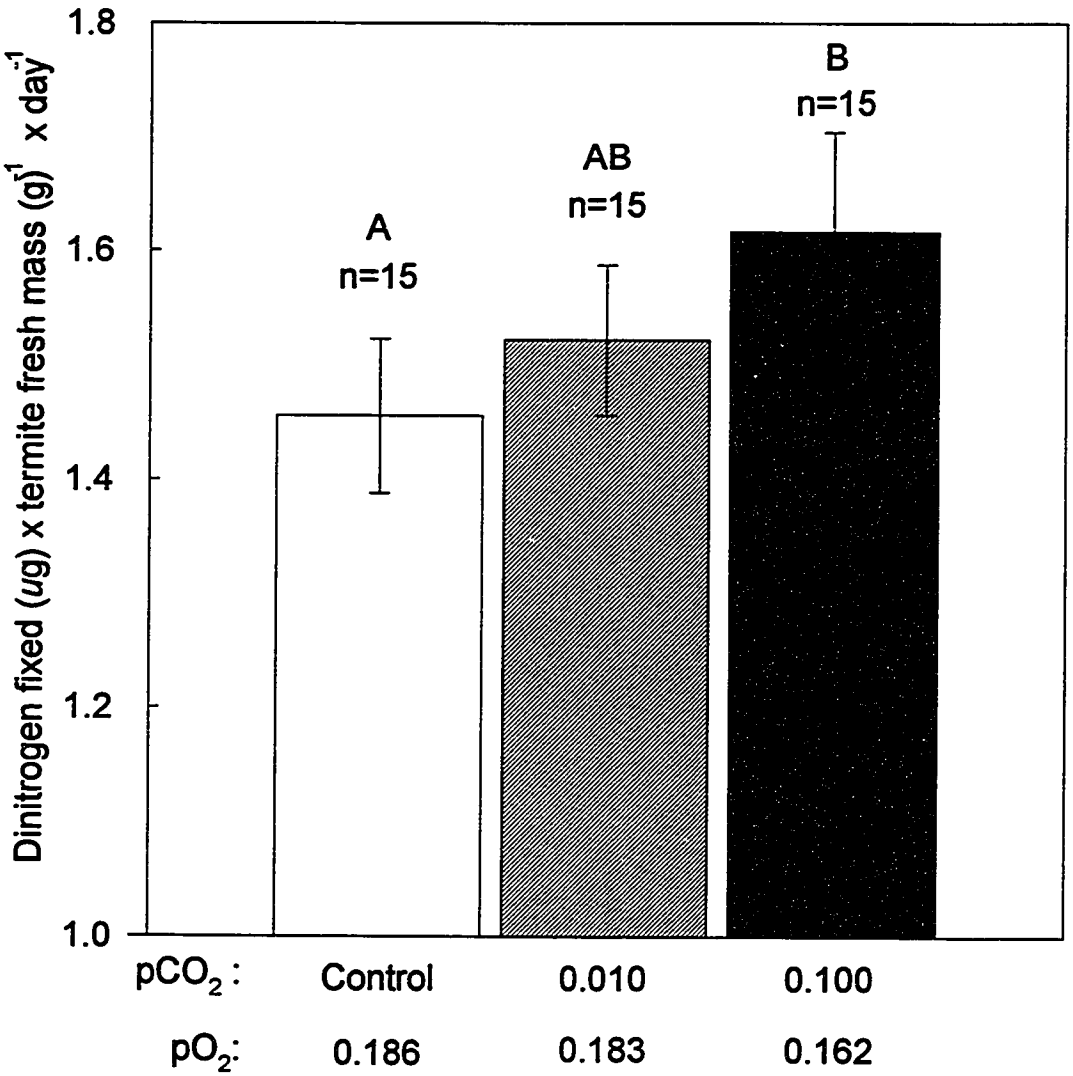

Figure 15. Nitrogen fixation rates for termites exposed to an atmosphere of $pCO_2 = 0.01$ and $pCO_2 = 0.1$. Bars with same letter are not significantly different at the 0.05 level.

Table 2. Summary of ANOVA tables for Chapter IV

Experiment I

Source	df	*F*	*P*
Total	44		
Colony	4	28.6	<0.0001
Treatment	2	4.24	0.0217
Error	38		

Experiment II

Source	df	*F*	*P*
Total	44		
Colony	4	7.77	<0.0001
Treatment	2	6.07	0.0052
Error	38		

Experiment III

Source	df	*F*	*P*
Total	29		
Colony	4	122	<0.0001
Treatment	1	0.72	0.4061
Error	24		

Experiment IV

Source	df	*F*	*P*
Total	74		
Colony	4	1.42	0.2372
Treatment	4	48.6	<0.0001
Error	66		

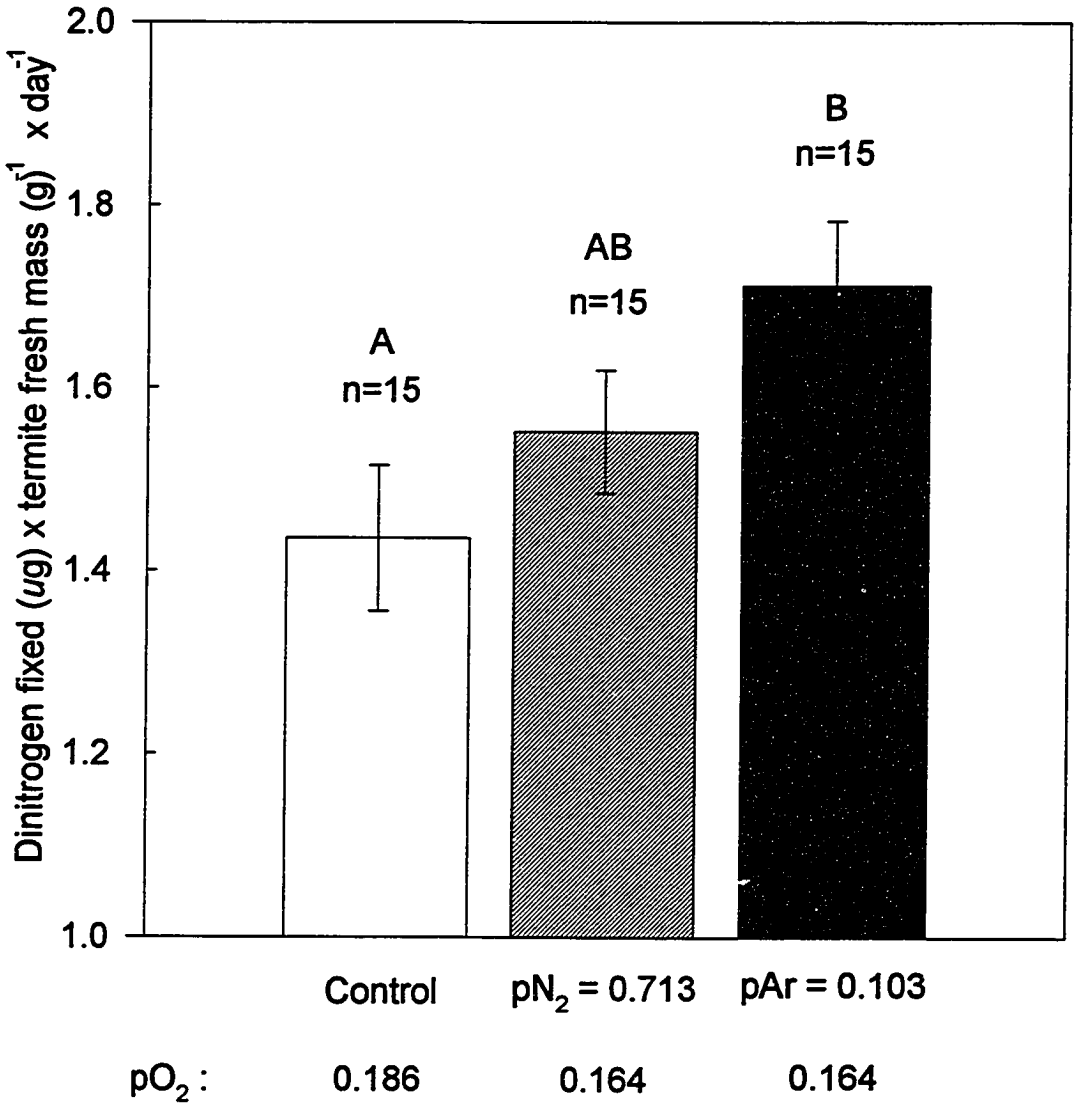

Figure 16. Nitrogen fixation rates for termites exposed to an atmosphere of $pO_2 = 0.186$ and $pO_2 = 0.164$. Bars with the same letter are not significantly different at the 0.05 level.

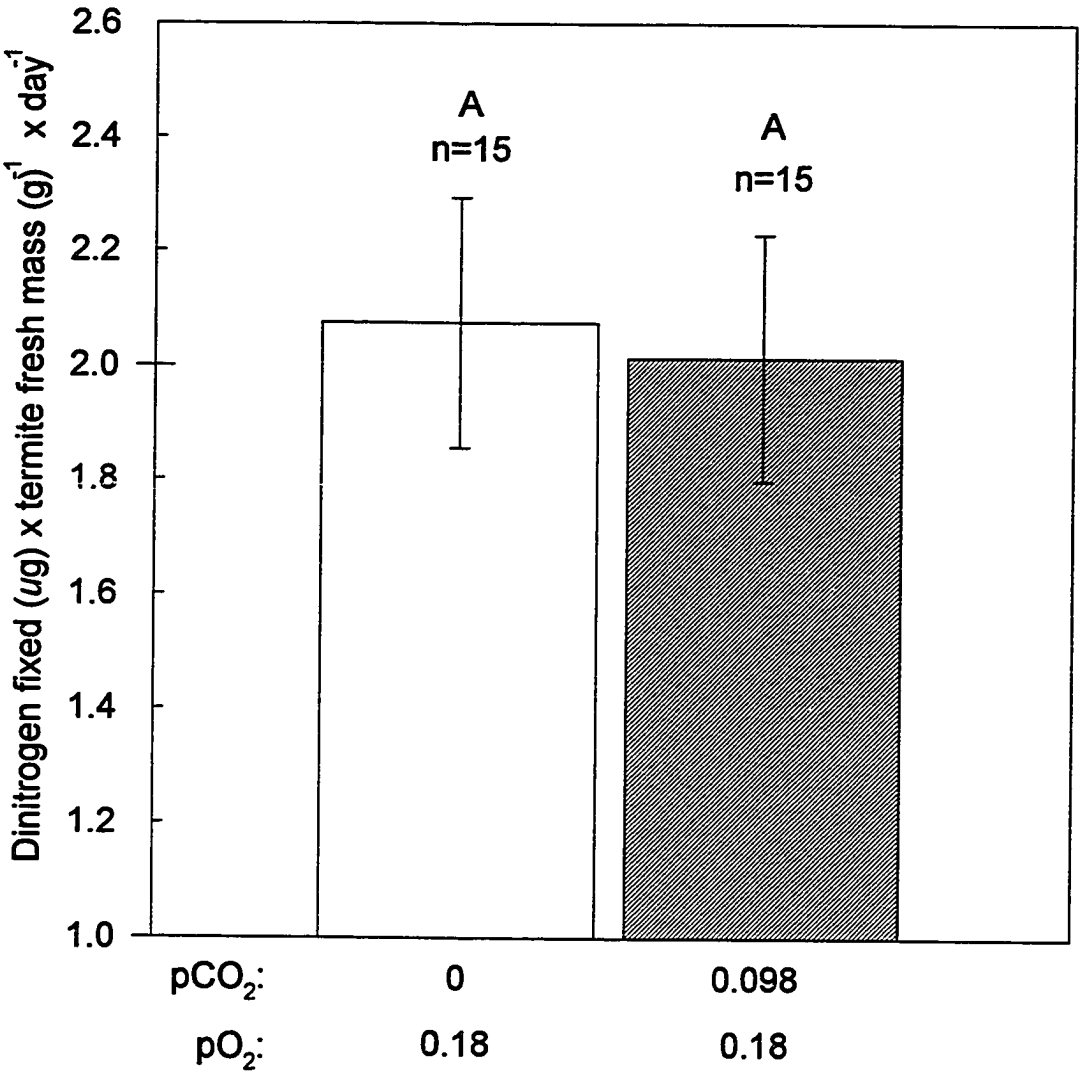

Figure 17. Nitrogen fixation rates for termites exposed to an atmosphere of pCO_2 = 0.098. Bars with the same letter are not significantly different at the 0.05 level.

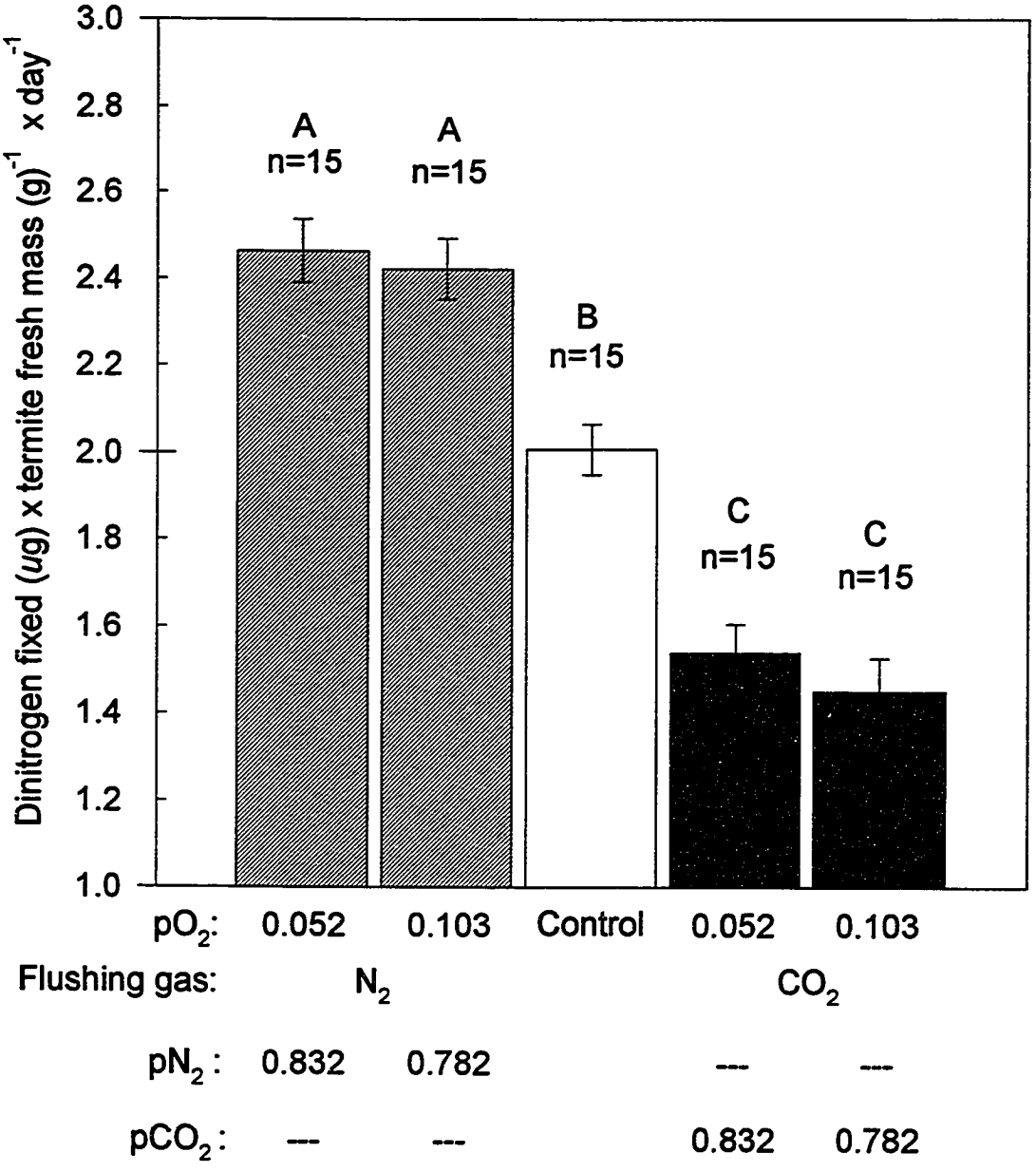

Figure 18. Nitrogen fixation rates for termites exposed to atmospheres of $pO_2 = 0.052$ and $pO_2 = 0.103$. Bars with same letter are not significantly different at the 0.05 level.

Discussion

In Experiment I, termite nitrogen fixation rates increased under high CO_2 and low O_2 conditions. However, it was unclear whether increased pCO_2 or decreased pO_2 levels were responsible. Because pO_2 decreased as pCO_2 increased for those treatments, we could not attribute the decrease in nitrogenase activity to the increased pCO_2 or the resulting decrease in pO_2. Experiment II tested the effect of decreased pO_2 independent of pCO_2. In this experiment, a decrease in pO_2 caused a significant increase in nitrogenase activity when 10.3% of the gas in the vials was replaced with argon, but not with N_2. This may have resulted because N_2 is a competitive inhibitor of nitrogenase. Since there was less N_2 in the argon treatment vials, perhaps the conversion of acetylene to ethylene proceeded slightly faster because there was less competition for active sites on the nitrogenase molecule. There may also be a potential inhibitory effect of acetylene on other microbial processes in the termite gut (Oremland 1988).

Many other nitrogen fixing organisms are inhibited by increased pO_2 (Fay 1992), but this is the first report of oxygen sensitivity in the termite-bacteria nitrogen fixing system. This finding is consistent with studies involving free-living cyanobacteria and symbiotic nitrogen fixation in plant nodules (reviewed in Fay 1992). Nitrogenase is inactivated by oxygen and its synthesis can be completely repressed (Postgate *et al.* 1981). Dalton and Postgate (1969) showed that nitrogen fixation rates are suboptimal at ambient atmospheric oxygen levels.

The enhanced termite nitrogen fixation rates at low pO_2 may be associated with the location of the N_2-fixing microbes in the gut if they are located at a site that can be

influenced by external pO_2. Brune *et al.* (1995) showed a steeply decreasing O_2 gradient from the exterior of the gut to the lumen which may be affected by changes in external pO_2. If the N_2-fixing bacteria were strictly anaerobic and located in the anoxic lumen of the termite hindgut, then changes in external pO_2 would not be expected to affect nitrogen fixation rates, except through indirect effects on the termite host.

Because the oxygen levels inside termite galleries may be lower than ambient atmospheric levels, and nitrogen fixation efficiency can be 10- to 20 times greater at $pO_2 = 0.01$ than at $pO_2 = 0.21$ (Burk 1930), the potential for underestimation of termite nitrogen contribution to ecosystems exists. Schaefer and Whitford (1981) estimated that *Gnathamitermes tubiformans* (Buckley) fix 66 g • ha^{-1} • yr^{-1} of atmospheric nitrogen in a desert ecosystem. However, this estimate was made using the acetylene reduction assay at ambient pO_2 ($pO_2 = .209$). The estimate by Pandey et al. (1992) of 125.5 - 445.3 g N fixed • ha^{-1} • yr^{-1} by *Reticulitermes* spp. in a forest ecosystem was based on assays performed at near ambient pO_2 levels ($pO_2 = 0.186$). Furthermore, the nitrogen fixation rates reported here are 20 to 50 times that reported by Breznak (1982). This suggests that some termites may be more important than previously realized in the biogeochemical cycling of nitrogen in some ecosystems. Therefore, termite nitrogen contributions based on nitrogen fixation assays conducted under ambient or near-ambient atmospheric conditions should be accepted as a minimal estimate.

Termite nests and galleries might also favor increased CO_2 levels (Matsumoto, 1977). Experiment III tested the effect of increased pCO_2 on nitrogenase activity while the pO_2 was held constant for both treatments. Under these conditions, termite

nitrogenase activity was not affected by an increase in pCO_2. Therefore, we attribute the increase in nitrogenase activity in Experiment I to the decreased pO_2 and not to the increased pCO_2. The decrease in termite nitrogen fixation rates for termites flushed with CO_2, in Experiment IV, is likely the result of a corresponding decrease in metabolic activity from CO_2 narcosis. Nitrogen fixation requires 12 to 15 mol of ATP per mol of N_2 fixed (Fay 1992). Because the termites were knocked-down for the duration of the assay (30 min), perhaps a reduction in ATP production resulted in an insufficient amount of energy for nitrogen fixation. Although the metabolic interactions between termites and their symbiotic nitrogen fixing bacteria are unknown, there are examples of decreased metabolic activity associated with increased pCO_2 in other organisms. Howarth (1983) attributes the low metabolic rate of cave-dwelling organisms to the high CO_2 levels of their cave habitats. Pache and Zachariassen (1973) report an increase in lactic acid in the haemolymph of beetles which experience high CO_2 levels in their overwintering chambers, indicating that oxidative phosphorylation is less than optimal.

Following Experiment IV, the rubber caps were removed from the vials and the CO_2-flushed termites regained mobility similar to that prior to the experiment. This experiment demonstrated that termites were capable of fixing nitrogen, albeit at a reduced rate, while experiencing CO_2 narcosis, which did not affect their mobility immediately following the experiment. In contrast, Potrikus and Breznak (1977) found that *Coptotermes formosanus* Shiraki lost nitrogenase activity when exposed briefly to an anoxic glove box containing $pN_2 = 0.90$ and $pH_2 = 0.10$.

These experiments demonstrate that termite nitrogen fixation rates increased when

termites were exposed to decreased pO_2 (pO_2 = 0.052, 0.103, and 0.162). Although increased pCO_2 (pCO_2 = 0.782 and 0.832) decreased nitrogen fixation rates in termites experiencing CO_2 narcosis, the nitrogenase activity of termites exposed to a subanesthetic level (pCO_2= 0.100) did not differ from ambient CO_2-level controls. Long-term studies are needed to determine the latent effects of decreased pO_2 and increased pCO_2 on termite nitrogen fixation so that the termite nitrogen contribution to ecosystems can be more accurately estimated.

CHAPTER V

CHANGES IN NITROGEN FIXATION RATES IN TERMITES MAINTAINED IN THE LABORATORY[3]

Introduction

Termites are important to forest ecosystems as consumers and detritivores (DeAngelis 1992). Termite microbial symbionts digest wood and contribute to the biogeochemical cycling of carbon, nitrogen, and other nutrients in the soil (Wood and Sands 1978, Waller and La Fage 1987a). The microbial gut flora of termites also include nitrogen-fixing bacteria (French *et al.* 1976, Potrikus and Breznak 1977), which may contribute substantial amounts of nitrogen to forests. However, the rate at which nitrogen is fixed varies among species (Prestwich *et al.* 1980, Breznak 1984, Bentley 1987, Waller *et al.* 1989), and nitrogen fixation activity varies intraspecifically with food quality (Prestwich *et al.* 1980), termite caste (Breznak *et al.* 1973, Hewitt *et al.* 1987), termite size (Waller *et al.* 1989), and seasonal factors (Schaefer and Whitford 1981, Waller *et al.* 1989). To understand fully the effect of termites on the nitrogen cycle in forest ecosystems, one must examine the factors that influence nitrogen fixation rates.

Previous studies have noted that nitrogen fixation rates decrease soon after termites are brought into the laboratory. Nitrogenase activity is lost in termites removed from

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[3]Material in this chapter was reprinted from "Curtis, A. D. and D. A. Waller. 1995. Changes in nitrogen fixation rates in termites (Isoptera: Rhinotermitidae) maintained in the laboratory. *Annals of the Entomological Society of America* 88: 764-767." and is copyrighted by the Entmological Society of America.

field nests (Prestwich *et al.* 1980), in whole nests removed from the field (Prestwich and Bentley 1981), and in termites maintained in the laboratory (Lovelock *et al.* 1985). Lovelock *et al.* (1985) reported that *Nasutitermes walkeri* (Hill) completely lost nitrogenase activity and did not recover it during the 11 d maintained in the laboratory. In the same study, *Nasutitermes exitiosus* (Hill) and *Coptotermes lacteus* (Froggatt) also lost the ability to fix atmospheric nitrogen and could recover only 25--50% of their original rates. The reasons for the decrease in nitrogen fixation rates under laboratory conditions remain unclear. Although loss of nitrogenase activity is common during confinement, nitrogen fixation rates might increase if the laboratory conditions were more favorable than the field conditions under which they were initially collected. For example, temperature might be involved because nitrogenase activity in the eastern subterranean termite, *Reticulitermes flavipes* (Kollar), and *R. virginicus* (Banks) is optimal at moderate temperatures and decreases at either temperature extreme in both the laboratory and the field (D.A.W. and A.D.C., unpublished data). In the current study, we examined whether termites collected in the field and maintained in the laboratory can recover and, if so, exceed their original rates of nitrogen fixation.

Materials and Methods

Termites. Wood infested with termites from 10 *Reticulitermes* spp. Holmgren colonies was collected bimonthly from December 1993 to June 1994 from pine forests near Nassawaddox on the eastern shore of Virginia. *R. flavipes* and *R. virginicus* are common to the site; however, the termites were not identified to species because there

were no alates. The samples were immediately returned to the laboratory, termites were removed from the wood, and nitrogenase activity was determined for each colony. The termites and wood pieces from each colony were then placed in 4.23-liter styrene containers and maintained at 22 $\pm$ 2 C for up to 6 mo. Voucher specimens from each colony have been deposited in the reference collection at Old Dominion University.

Nitrogenase Activity. The acetylene reduction bioassay was used to determine nitrogen fixation rates for each termite colony. The assay is based on the ability of nitrogenase to reduce acetylene to ethylene at 3 times the rate that it reduces dinitrogen to ammonia (Hardy *et al.* 1973, Bentley 1984). The following protocol is adapted from Pandey *et al.* (1992).

Fifty worker termites were removed from their nest material and weighed to the nearest 0.1 mg. They were placed in an 8.5-ml glass vial with a rubber sleeve cap septum. One ml of air was replaced by 1.0 ml of acetylene, resulting in a final atmosphere of $\approx$12% acetylene. Three replicate vials per colony were incubated at 22 $\pm$ 2^{0}C for 30 min. Following incubation, a 200-μl sample of head space was removed with a 0.5 ml Hamilton gas tight syringe (Hamilton Company, Reno, Nevada) and injected into a Varian 3600 gas chromatograph (Varian Instrument Group, Walnut Creek, California) equipped with a flame ionization detector and a Porapak N column (Alltech, Deerfield, Illinois). Ethylene peaks were analyzed using a standard concentration curve with known amounts of ethylene to determine the moles of ethylene produced for each sample. Final rates are expressed as N_2-fixed (μg) $\cdot$ termite fresh mass $(g)^{-1} \cdot day^{-1}$.

Initial nitrogenase activity was determined for 10 field colonies collected in

December 1993, February 1994, April 1994, and June 1994. All surviving colonies were assayed for nitrogenase activity again in June 1994. These colonies had been maintained in the laboratory for 3 wk, 2 mo, 4 mo, and 6 mo, respectively.

Statistical Analysis. Not all initial colonies survived to be sampled again in June 1994. A randomized complete block design analysis of variance (ANOVA) was used to partition surviving colony effects and to compare the initial termite nitrogenase activity with the nitrogenase activity of the same colonies maintained in the laboratory. To test for a difference among the initial nitrogenase activities from December 1993 to June 1994, a completely randomized design ANOVA was used; and the Tukey honestly significant difference (HSD) multiple comparison test was used to compare means (SAS Institute, 1990).

Results and Discussion

The initial nitrogen fixation rates of termites from the 6 colonies collected in June 1994 were significantly higher ($F = 13.82$; df = 1, 29; $P < 0.001$) (Table 3) than the nitrogen fixation rates of the same colonies maintained in the laboratory for 3 wk (Fig. 19). This 21% loss in nitrogenase activity is similar to earlier observations of termites maintained in the laboratory. Lovelock *et al.* (1985) reported a loss of 85% of original nitrogenase activity in 2 colonies of *N. exitiosus* also kept for 3 wk in the laboratory.

In contrast, we observed that nitrogen fixation rates of termites kept in the laboratory for 2 mo did not differ ($F = 2.21$; df = 1, 34; $P = 0.15$) (Table 3) from the rates initially measured for the same colonies in April 1994 (Fig. 19).

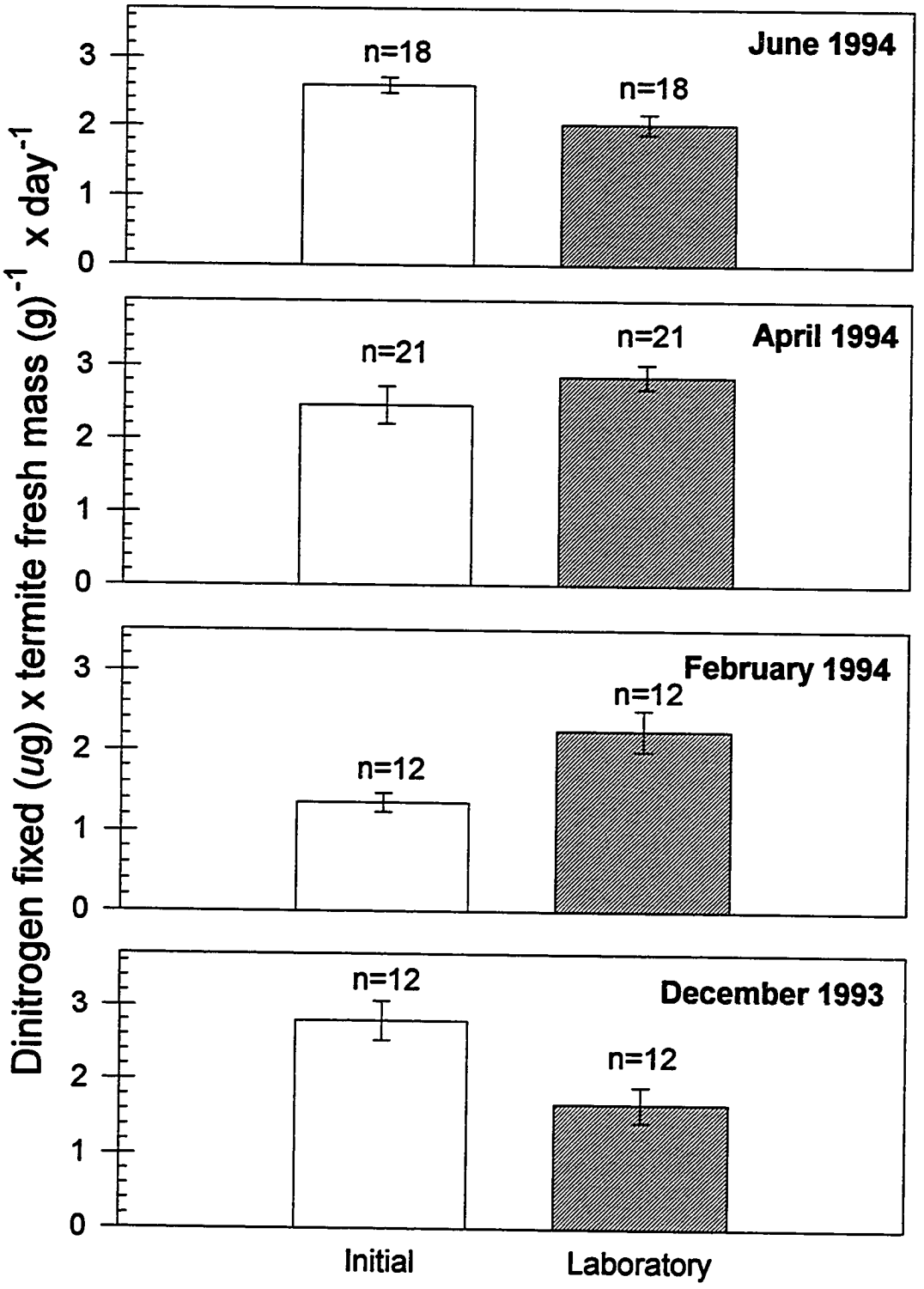

Figure 19. Initial nitrogen fixation rates of termites collected from field colonies and nitrogen fixation rates of the same colonies after laboratory containment.

Table 3. Summary of ANOVA tables for Chapter V

June 1994

Source	df	*F*	*P*
Total	35		
Colony	5	4.34	0.0046
Treatment	1	13.82	0.0009
Error	29		

April 1994

Source	df	*F*	*P*
Total	41		
Colony	6	3.38	0.01
Treatment	1	18.55	0.1465
Error	34		

February 1994

Source	df	*F*	*P*
Total	23		
Colony	3	7.29	0.0019
Treatment	1	18.55	0.0004
Error	19		

December 1993

Source	df	*F*	*P*
Total	23		
Colony	3	48.67	<0.0001
Treatment	1	72.79	<0.0001
Error	19		

Initial Field Rates

Source	df	*F*	*P*
Total	62		
Treatment	3	7.02	0.0004
Error	59		

The most dramatic change in nitrogenase activity occurred in termites collected in February 1994 and maintained in the laboratory for 4 mo. Nitrogen fixation rates increased 67% ($F = 18.55$; df = 1, 19; $P < 0.001$) (Table 3) over that time (Fig. 19; Table 4). This is the 1st report of termites maintained in the laboratory exceeding their initial nitrogen fixation rates.

The increase is probably related to the season in which the termites were collected and assayed for nitrogenase activity; February 1994, had a monthly mean air temperature of 3.5 C (Krovetz and Porter 1994) (Table 4). It is likely that the resulting increase in nitrogenase activity was influenced by the initial condition of the termites. In an unpublished experiment, termites exposed to 26 C had higher nitrogen fixation rates after 3 wk than did termites maintained at 22 and 32 C for the same length of time (D.A.W. and A.D.C., unpublished data). Therefore, if the termites are collected and assayed during unfavorable temperatures, maintaining them in the laboratory may result in an increase in nitrogenase activity.

Nitrogenase activity of termite colonies maintained in the laboratory for 6 mo was significantly lower ($F = 72.79$; df = 1, 19; $P < 0.0001$) (Table 3) than the activity at the time of their collection in December 1993 (Fig. 19). Although the mean monthly temperature in December was only 4.1 C, we sampled termites at the beginning of the month, and the previous monthly temperatures were moderate.

There was a significant difference among the initial nitrogen fixation rates of termites collected in December 1993, February 1994, April 1994, and June 1994 ($F = 7.02$; df = 3, 59; $P< 0.001$) (Table 3) (Fig. 19). The rates of termites collected in

Table 4. Summary of collection month, laboratory containment time, the change in nitrogen fixation rates (mean difference $\pm$ SE), mean monthly air temperature, and sample size

Collection month	Laboratory containment time	Change in nitrogen fixation rates	Mean monthly air temp, ^{0}C	Sample size
June 1994	3 wk	-0.6 $\pm$ 0.15*	22.7	36
April 1994	2 mo	+0.4 $\pm$ 0.27NS	15.7	42
Feb. 1994	4 mo	+0.8 $\pm$ 0.21*	3.5	24
Dec. 1994	6 mo	-1.1 $\pm$ 0.13*	4.1	24

Units for nitrogen fixation rates are N_2-fixed (μg) · termite fresh mass (g)$^{-1}$ · day^{-1}.
*P-value $<$ 0.001. NS, not significant.

February 1994 were lower than those of termites collected at other times (Tukey HSD).

Decrease in termite nitrogenase activity following laboratory containment is believed to be the result of nest disturbance during collection (Prestwich *et al.* 1980, Prestwich and Bentley 1981, Lovelock *et al.* 1985). Although it is not known precisely why these termites lose nitrogenase activity, their ability to regain or exceed their initial rates has not been demonstrated until now.

Because there is an apparent seasonal component to termite nitrogenase activity, estimates of nitrogen contributed to forest ecosystems should account for this variability. Long-term studies with monthly observations are needed to describe more accurately termite nitrogen inputs to forests.

In conclusion, the length of confinement alone plays little role in the ability of termites to fix nitrogen. Initial environmental conditions, however, appear to have the greatest effect on whether nitrogenase activity increases or decreases following laboratory confinement. Therefore, it will be important for future studies to consider seasonal environmental influences on the dynamics of nitrogenase activity in termites.

CHAPTER VI

MARK-RELEASE-RECAPTURE IN SUBTERRANEAN TERMITES USING INGESTIBLE DYE MARKERS

Introduction

Estimating the foraging population of subterranean termite colonies is difficult because of their cryptic behavior (Forschler 1994). Termites can tunnel several meters below the surface of the soil (Watson 1960) and can forage over more than a thousand square meters (Grace *et al.* 1989, Grace 1990, Su *et al.* 1993). Although direct counts are sometimes possible (Howard *et al.* 1982, Nutting and Jones 1990), such destructive sampling techniques preclude further study of the termite colony. Using mark-release-recapture studies to estimate subterranean termite colony size allows future monitoring of population dynamics for pest management and ecological study purposes.

The Lincoln index (Peterson method) is commonly used to determine the size of field populations of subterranean termites (Grace *et al.* 1989, Grace 1990, Su *et al.* 1984). However, there is usually a large standard error associated with these estimates. Su and Scheffrahn (1988) and Su *et al.* (1993) used the triple-catch method with weighted means (Begon 1979) instead of the simple Lincoln index to measure termite colony size, thereby improving the precision of the population estimates by reducing the standard error of the estimate (Oi and Su 1994). However, estimates may be precise but not accurate. The accuracy of both techniques is based on the same five assumptions: 1) the population

is closed, 2) all animals have the same chance of being caught, 3) marking does not affect their catchability, 4) animals do not lose their marks, and 5) all marks are reported in the recapture sample (Begon 1978, Krebs 1989). Estimate bias may arise if one or more of these assumptions is violated.

In the present study, we adapted the mesocosm concept (Odum 1984) to determine the accuracy and precision of the population estimates generated by the Lincoln index. Mesocosm refers to an experimental design which bridges the gap between the laboratory and the real world environment. In the laboratory, termites were maintained in their host logs collected from the field. The termite population for each log was first estimated by the Lincoln index, and then the termites were directly counted for comparison with the estimate. We tested two concentrations of the dye marker Nile blue: 0.05% which is routinely used in mark-release-recapture studies in *Reticulitermes* spp. (Su *et al.* 1993, Haagsma and Rust 1995, Forschler and Henderson 1995) and a concentration of 0.1% for comparison. Previous laboratory studies have shown that termite mortality is greater with 0.1% Nile blue than at 0.05% (Haagsma and Rust 1993).

Materials and Methods

Pine logs infested with *Reticulitermes virginicus* (Banks) were collected from southeastern Virginia from July, 1995 through September, 1995. Voucher specimens from each colony were deposited in the reference collection at Old Dominion University. Each log was approximately 70 cm long x 25 cm in diameter and each was isolated in a 20-gallon metal trash can. Termites were removed from a section approximately 1/8 the

mass of each log and placed into a 1-gallon plastic container. The containers were first prepared by adding 200 ml of vermiculite, 100 ml of deionized water, and 6 paper towels dyed with either 0.05% or 0.1% (w/w) Nile blue. After 5 days, only dyed termites were counted and then released into their respective logs.

One week later, another 1/8 section of the log was removed and the marked and unmarked termites were counted. Immediately following this recapture count, the remaining portion of the log was broken and all termites were removed using an 8-mesh wire sifter. Their total volume was measured, and 10% of the total volume of termites was counted in three equal replicates. Termite population size was determined by multiplying the total volume of termites by the average number of termites per milliliter of the three replicates.

Results

The population estimates from the colonies fed 0.05% Nile blue were significantly higher ($F = 13.9$; $df = 1,4$; $P = 0.020$) than the population estimates for termites fed 0.1% Nile blue (Fig. 20). Estimates using the 0.05% concentration ranged from 7 to 12 times higher than the actual count, while estimates using the 0.1% concentration ranged from 1.6 to 3 times higher than the actual number of termites in the log (Table 5). Standard errors were also lower for the 0.1% concentration (0.5% to 7.5% SEM) than for the 0.05% concentration (5.5% to 16.6% SEM). However, even low standard errors resulted in large overestimations of population size. At the 0.05% concentration, our population was overestimated 12 times, even though there was a 5.5% SEM. At the

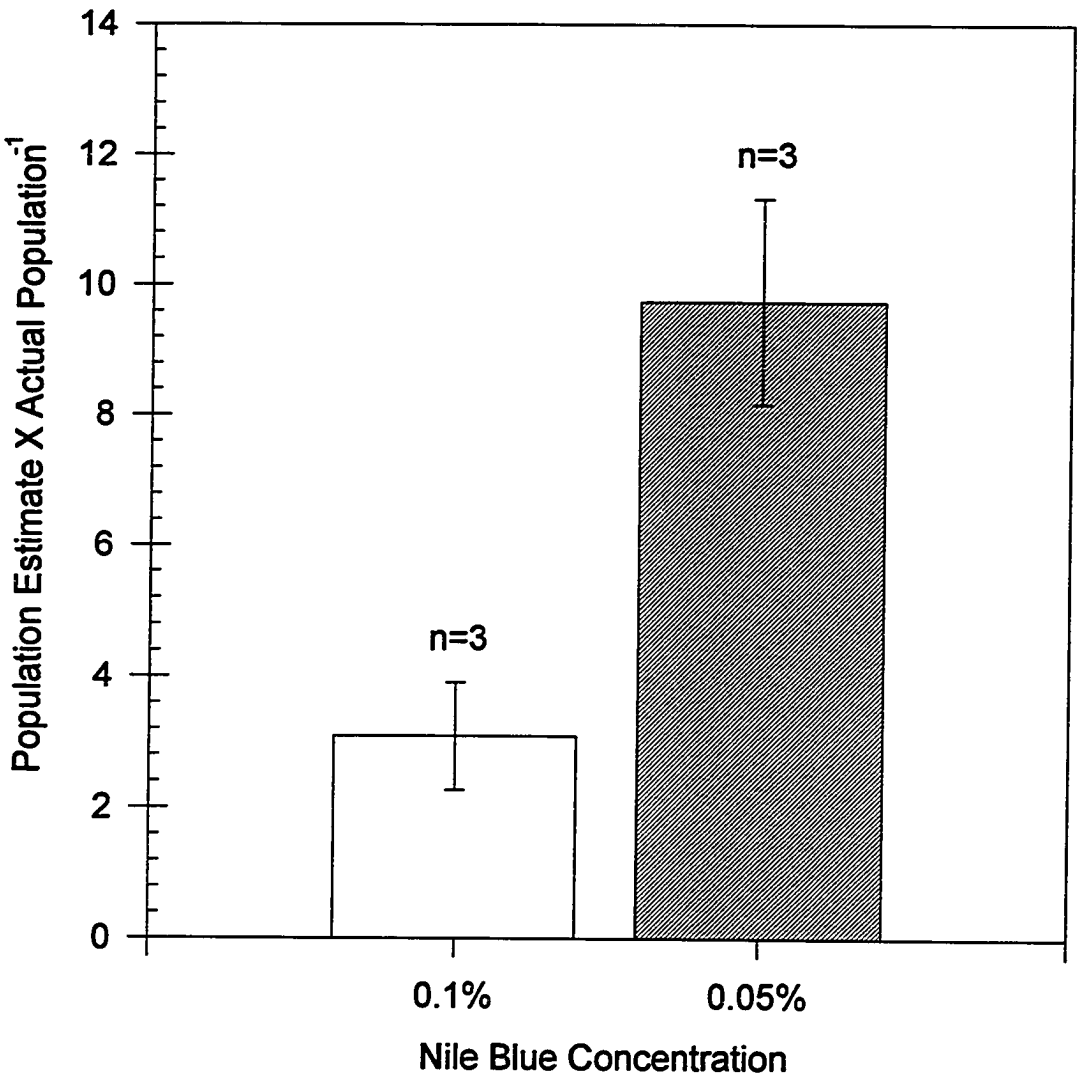

Figure 20. Termite population estimates of laboratory colonies.

Table 5. Lincoln index estimates of termite colony population size and the actual population size determined by direct count for both concentrations of Nile blue.

Nile blue conc. (w/w)	Colony	Population estimate*	SEM**	% SEM	Actual population	estimate X actual^{-1}
0.05%	1	82845	±13727	16.6%	11705	7
	2	527449	±29169	5.5%	42030	12
	3	95747	±11914	12.4%	9953	9.6
0.1%	4	131799	±2245	1.7%	78107	1.6
	5	62586	±4688	7.5%	13798	4.5
	6	203595	±998	0.5%	66970	3

*Lincoln index estimate

**Standard error associated with the Lincoln index estimate

0.1% concentration, one population was overestimated 3-fold, even though there was a 0.5% SEM (Table 5).

Discussion

There are generally two classes of bias that researchers may encounter with any mark-recapture method: small sample bias and model bias (White *et al.* 1982). Small sample bias for the Lincoln index is easily avoided as sample size increases, or it can be overcome by the use of an unbiased estimator (Bailey 1952). Model bias is a more serious problem, and it arises when the model is based on assumptions that are incorrect.

There are five assumptions that must be met for the Lincoln index to be effective: 1) the population is closed, 2) all animals have the same chance of being captured in the first sample, 3) marking individuals does not affect their catchability, 4) animals do not lose their marks between sampling periods, and 5) all marks are reported on discovery in the second sample (Krebs 1989). All of these can be violated in the study of termite populations in the field if only laboratory data are used for the selection of the dye markers.

The first assumption of a closed population requires that there is neither emigration nor death in the population. To avoid violating this assumption the time between release and recapture should be kept to a minimum, allowing only enough time for complete mixing (Begon 1978). This assumption goes relatively unchallenged in small-scale laboratory settings because there are physical boundaries for the experimental units and conditions are nearly ideal for termite survival (e.g temperature, relative humidity, ample

food). In the field, however, conditions may be less than optimal, and death may result from climate changes or predation. If the mortality rate in a colony is high, especially for marked individuals, the Lincoln index would tend to overestimate the foraging population. Because there were no predators or the possibility of emigration in our experimental units, the results indicate that there may be a high mortality rate for termites at both Nile blue concentrations. In our units, the average mortality of dyed workers was 79.6% $\pm$ 3.4% for 0.1% and 91.4% $\pm$ 3.7% for 0.05% Nile blue concentration.

The difficulty in the interpretation of the data is that the observed mortality cannot be distinguished from either an effect of the dye, the natural mortality rate of termites in the laboratory, or cannibalism of dyed workers which may occur in the field or the laboratory. In any case, the assumption of a closed population is violated, and the population estimate by the Lincoln index or any other method which assumes a closed population, including the triple-catch method, should be accepted as a maximum estimate of the population.

The second assumption of equal catchability in the first sample is somewhat less restrictive for social insects than nonsocial organisms because it is understood that only the foraging population can be estimated due to the division of labor within a colony (Ayre 1962). However, this assumption raises some question as to the usefulness of mark-recapture models that rely on random distribution to accurately estimate population size; termites may not forage randomly.

The third assumption that marking does not affect catchability may be more important than previously thought. There is some evidence that termites may be affected

by marking with ingestible dyes. *R. virginicus* fed 0.1- and 0.25% (w/w) Sudan IV appeared to be less vigorous than controls (Oi and Su 1994). The nitrogenase activity of *Reticulitermes flavipes* fed 0.2% (w/w) Nile blue is significantly less than termites fed water-treated controls (Curtis unpublished data). Grace and Abdallay (1989) showed *R. flavipes* fed significantly less on papers dyed with Sudan Red 7B (Fat Red 7B) than controls over 15 days. Furthermore, the synergism of two or more dyes may affect termite behavior or survivability. Synergistic effects have not been investigated, but in some field studies termites were marked with a different color dye after they had been previously marked (Haagsma and Rust 1995). Because there is a dose response mortality in termites fed single dye markers (Su *et al.* 1991), feeding termites multiple dyes may intensify this response. If a sublethal dose of dye marker affects the behavior or metabolic processes of the termites, then their catchability may also be affected which could lead to an inaccurate population estimate.

The fourth and fifth assumptions that marks are not lost and that all marks are reported have particular relevance to the present study. We tested two concentrations of Nile blue, 0.05- and 0.1% (w/w). The population estimates from the colonies fed 0.05% Nile blue were significantly less accurate than the population estimates for termites fed 0.1% Nile blue. The population estimates of termites fed 0.1% Nile blue were on average only $\approx$ 3 times the actual population. In contrast, the population estimates of termites fed 0.05% Nile blue were on average $\approx$ 10 times greater than the actual population. Termites fed 0.05% Nile blue dyed paper were noticeably lighter in color than those stained with 0.1% Nile blue immediately following the marking step. Over

the week between release and recapture of the dyed termites, their color faded considerably. One explanation for the overestimation is that the color faded in the 0.05% colonies to a point which rendered some of them undetectable upon recapture. Su and Scheffrahn (1988) reported the population of *Coptotermes formosanus* Shiraki, using the triple-catch weighted-mean method, to be 10 times greater than direct counts previously reported for that species. If the termites lose their marks in the field, then the estimate would be larger than the actual population. Although laboratory studies found termites fed 0.1% (w/w) Nile blue suffered greater mortality than those fed 0.05%, we found greater accuracy with the higher concentration marker, probably because the mark lasted longer.

In this study, termites fed on dyed paper for 5 days, but the length of time that termites are allowed to feed on dyed papers varies from 3 to 10 days (Forschler and Henderson 1995, Grace *et al.* 1989, Grace *et al.* 1995, Haagsma and Rust 1995, Oi and Su 1994, Su and Scheffrahn 1988, Su *et al.* 1991, Su *et al.* 1993). Subterranean termites that have been fed 0.05% and 0.25% (w/w) Nile blue for three days have been reported to be distinguishable from controls for up to 15 days (Su *et al.* 1991). However, those termites were compared to termites fed white filter paper and maintained in petri dishes following the marking step. In this experiment, termites were returned to their host logs following marking. Perhaps the dye dissipates faster under the more natural conditions inside the log. Another explanation for the observed fading of the dye is that there may be decreased visible contrast between termite guts with dyed paper mixed with wood particles compared to those with dyed paper mixed with undyed paper. This would make

it more difficult to distinguish marked from unmarked individuals in the field resulting in fewer marked individuals being recorded. Therefore, the population would be overestimated.

Although the triple-catch weighted-mean method (Begon 1978) can sometimes reduce the standard error of population estimates, the validity of its accuracy is dependent on the assumptions made. For example, in our study the simple Lincoln index provided lower standard errors than those reported by Su and Scheffrahn (1988), Su *et al.* (1993), Forschler and Henderson (1995), and Haagsma and Rust (1995) using the triple-catch weighted mean method (Table 5). However, the accuracy of our estimate was as much as 12 times greater than the actual count of termites (Table 5). Therefore, the estimate is very precise but not very accurate. The validity of the population estimate of the triple-catch weighted-mean method is based on the same assumptions as the simple Lincoln index (Begon 1978), and therefore it is possible to produce very precise although not very accurate population estimates due to model bias using either method (White *et al.* 1982).

Many dye markers have been laboratory tested for their usefulness in termite mark-release-recapture studies, but only a few have been accepted by researchers (Su *et al.* 1983, Su *et al.* 1988, Su *et al.* 1991, Grace and Abdallay 1990). Nile blue is a dye marker that has been recommended for studies on *Reticulitermes* spp. (Su *et al.* 1983, Su *et al.* 1991, Haagsma and Rust 1993). However, mortality rates have only been measured under laboratory conditions, and our results indicate that dye concentrations causing higher mortality in the laboratory are more accurate in estimating population size.

The difficulty of measuring termite mortality in field populations is obvious and may require further mesocosm approaches to approximate field conditions to understand the population dynamics of subterranean termites. The experimental units used here are more similar to field conditions than those of the typical laboratory set-up and may provide a more realistic environment under which to test the efficacy of dye markers. If the assumptions governing the accuracy of the techniques are violated, then the validity of the estimates should be tempered accordingly.

CHAPTER VII

CONCLUSION

Termites are important to the biogeochemical cycling of carbon in forest ecosystems through their roles as detritivores. The ability of termites to fix atmospheric nitrogen also makes them important in the biogeochemical cycling of nitrogen in these ecosystems. To calculate the annual nitrogen input by termites per unit area, several factors must be considered. These include 1) seasonal variation in termite nitrogen fixation rates, 2) nitrogenase activity of different termite castes related to the seasonal abundance of those castes, 3) the amount of nitrogen consumed by termites and the effect of dietary nitrogen on nitrogenase activity, 4) the potential effect of low oxygen conditions within termite nests and subterranean galleries, 5) how laboratory containment affects nitrogenase activity, 6) and how accurate are current methods employed in estimating termite abundance.

Termite nitrogen fixation rates were related to the temperature at which the colony was conditioned. Rates were highest during moderate temperatures and were lowest at either temperature extreme. Termite fresh mass, dry mass, and body water were also dependent on the season in which they were measured. Because there is seasonal variation in termite nitrogen fixation rates, estimates of termite nitrogen contribution must account for this variability.

The nutrient status of termite colonies may influence termite nitrogen fixation

rates. There were significant differences in the nitrogen content of natural termite food. Although we found no correlation between termite nitrogenase activity and natural dietary nitrogen in our field studies, rates were depressed when termites were maintained on certain nitrogen sources in laboratory studies. In other nitrogen fixing systems it is generally accepted that nitrogen fixation rates decrease as available nitrogen increases (Sprent 1979, Postgate 1982). However, our studies show that the relationship between dietary nitrogen and termite nitrogen fixation cannot be generalized in this way.

Termite nitrogen fixation rates are sensitive to changes in oxygen concentration. This is the first report of oxygen sensitivity to be demonstrated in the termite nitrogen fixing system. Because termites may live deep within logs and in soil, the oxygen concentration to which they are exposed may be less than ambient levels. By measuring nitrogenase activity at near ambient atmospheric conditions, termite nitrogen contributions may be underestimated.

Previous studies indicated that termites lose their nitrogen fixing ability days after being brought into the laboratory (Lovelock *et al.* 1985). Our data demonstrate that termites, in the laboratory, may regain or exceed their original nitrogen fixation rates, depending on the environmental conditions at the time they are collected. For example, the initial nitrogenase activity of termites that were collected during the winter were low. However, after they were maintained in the laboratory for a few months their nitrogenase activity increased beyond their initial rates. This is the first report of laboratory maintained termites exceeding their initial nitrogen fixation rates.

Finally, estimating termite abundance is an important step in determining termite

nitrogen contributions to ecosystems. Because of their cryptic behavior, termite populations are among the most difficult to quantify. The Lincoln index (Peterson method) is frequently used in animal population studies. Many researchers have used the technique to study termite populations for pest management and ecological applications. The accuracy of the Lincoln index is based on critical assumptions about the population to be estimated. Recently, other mark-recapture methods, such as the triple-catch method, have been used to study termite populations because they have been shown to increase the precision of the estimate by lowering the standard errors. However, it is possible to have a very precise but not very accurate estimate. For example, our laboratory study demonstrated that even with very low standard errors, the population size may be overestimated by several times. The accuracy of these other methods is based on the same assumptions as the Lincoln index. If the assumptions are violated then the population estimate may be biased.

Laboratory experiments were designed to test the accuracy of the Lincoln index. Termite populations maintained in the laboratory were estimated by the Lincoln index and then the termites were directly counted for comparison with the estimate. The results suggest that a higher dye concentration may be preferred by researchers because it was more accurate.

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APPENDICES

Permission letter from the *Journal of Insect Physiology*

Journal of Insect Physiology

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4 March 1996

Mr. Anthony D. Curtis
Department of Biological Sciences
Old Dominion University
Norfolk, VA 23529

RE: "The effects of decreased pO_2 and increased pCO_2 on nitrogen fixation rates in termites (Isoptera: Rhinotermitidae)" by CURTIS, A. D. and WALLER, D. A.

Dear Mr. Curtis:

The above manuscript was recently accepted by me for publication in the *Journal of Insect Physiology*. I'm pleased to note that this represents a chapter from your Ph.D. thesis, and you are quite free to have this manuscript included as part of your thesis.

Wishing you the best of success as you complete your degree requirements.

Sincerely,

David L. Denlinger
Editor

DLD/isl

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Permission letter from the *Annals of the Entomological Society of America*

ENTOMOLOGICAL SOCIETY OF AMERICA

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March 15, 1996

Dr. Anthony Curtis
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Norfolk, VA 23529

Dear Dr. Curtis:

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Sincerely,

Ray Everngam
Assistant Director,
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PRESENTATIONS AT PROFESSIONAL MEETINGS

Curtis, A. D. and D. A. Waller. 1995. Field and laboratory dietary nitrogen effects on nitrogen fixation rates in Reticulitermes (Isoptera: Rhinotermitidae). 80th Annual Meeting of the Ecological Society of America, Snowbird, Utah.

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in press

Curtis, A. D. and D. A. Waller. 1996. The effects of decreased pO_2 and increased pCO_2 on nitrogen fixation rates in termites (Isoptera: Rhinotermitidae). J. Insect Phys.