Effect of Enhanced Dietary Nitrogen on Reproductive Maturation of the Termite *Zootermopsis angusticollis* (Isoptera: Termopsidae)

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ABSTRACT   We determined the effect of enhanced dietary nitrogen on the ovarian maturation of female primary and neotenic reproductives of the termite *Zootermopsis angusticollis* Hagen. Supplementing the wood diet of newly paired reproductives with a 0.05% uric acid solution resulted in both primaries and neotenics gaining less body mass. This may have occurred because the increased nitrogen content of their food allowed reproductives to consume less wood to meet their dietary needs, thereby reducing the mass of their gut contents. An abundance of exogenous nitrogen may have also stimulated females to excrete excess uric acid rather than store it, further reducing mass gain. Nitrogen supplementation resulted in significant increases in ovariole number and fecundity for neotenic females but not primary females. These results suggest that although enhancing dietary nitrogen may release newly molted neotenics from nutritional limitations on their fecundity, dietary enhancement with 0.05% uric acid does not significantly effect the reproductive development of recently dealated primaries. Possible reasons for each reproductive form's response to enhanced dietary nitrogen are discussed.

KEY WORDS   *Zootermopsis angusticollis*, nitrogen limitation, reproduction, termites, ovaries, fecundity

INSECT OVARIAN DEVELOPMENT and oogenesis is often regulated by nutrition (Johansson 1964, Norris 1964, Engelmann 1970, Wilson 1971, McCaffery 1975, Hölldobler and Wilson 1990, Hunt 1991, Lenz 1994). Nitrogen availability is particularly important for reproduction in termites, which feed on a nutritionally poor food source (LaFage and Nutting 1978, Waller and LaFage 1987). Although wood is an excellent source of carbohydrates (Cook and Scott 1933, Moore 1969) it contains a low concentration of nitrogen (Cowling and Merrill 1966, LaFage and Nutting 1978), which is necessary for the production of vitellogenins (Leather 1995). To ensure colony growth, the C/N ratio must be balanced to meet the needs of growth and reproduction; in wood-feeding termites, one option is to selectively increase the intake of nitrogen (Collins 1983, Waller and LaFage 1987, Higashi et al. 1992, Shellman-Reeve 1994). Shellman-Reeve (1990) showed that the probability of oviposition increased when primary females of the termite *Zootermopsis nevadensis* (Hagen) were maintained on a diet with supplemental nitrogen in the form of uric acid. Although termites in that study were provided a diet that did not contain all of the essential nutrients necessary for normal development (Cook and Scott 1933, Honigberg 1970), results suggest that nitrogen availability can influence female reproduction. Similarly, colonies of *Z. angus-ticollis*, *Z. nevadensis* (Hungate 1941), and *Kalotermes flavicollis* F. (Hungate 1941, Becker 1965) grow faster when fed wood with enhanced nitrogen content than when fed sound wood.

A primary reproductive may maximize access to nitrogen by nest site selection during colony foundation (Nutting 1969, Abe 1987, Waller and LaFage 1987, Waller et al. 1990, Shellman-Reeve 1994, Traniello and Leuthold 2000) and by metabolizing her well-developed fat body and flight muscles to obtain the resources needed to complete reproductive maturation and initiate oogenesis (Grassé 1949, Nutting 1969). Primaries also begin their reproductive lives with endogenous reserves of uric acid (Shellman-Reeve 1990), which can be reduced by uricolytic bacteria in the hindgut, releasing energy and nitrogen to directly enhance reproductive potential (Potrikus and Breznak 1981; Shellman-Reeve 1990, 1996). Neotenic reproductives, in contrast, normally develop in established colonies and thus do not select their food source (Miller 1969). Because neotenics arise directly from larvae and have few stored resources relative to primaries, their nitrogen resources may be derived largely from their own feeding and in secretions provided by trophallaxis with nestmates (McMahan 1969, LaFage and Nutting 1978, Breznak 1982, Shellman-Reeve 1990, Rosengaus and Traniello 1993, Nalepa 1994, Machida et al. 2001).

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In the current study we supplemented the diets of primary and neotenic reproductives of the dampwood termite *Zootermopsis angusticollis* (Hagen) with uric acid. We measured the physiological responses of primary and neotenic female fecundity to standardized and nitrogen supplemented diets to determine whether either of these reproductive forms may be nitrogen limited when they first begin to produce eggs. We predicted that a nitrogen enhanced diet would promote ovarian development in both primary and neotenic females, but that supplemental nitrogen should have a greater impact on the fecundity of neotenics due to their initially greater reliance on exogenous sources of nitrogen.

Materials and Methods

Termites originated from four stock colonies of *Zootermopsis angusticollis* collected in 1996 from the Redwood East Bay Regional Park, near Oakland, CA. Stock colonies were maintained in wood in which they were collected. Termites from these colonies were tested within a few months of their being collected. Primary reproductives were selected from alates showing flight behavior. Neotenics were generated from groups of female and male fourth- to sixth-instar larvae isolated from their parent colonies in clear covered plastic boxes (17 by 12 by 6 cm) containing moistened filter paper and wood from their natal nest. Neotenics were collected within 1–3 d of their adult molt, as determined by their darkening pigmentation. Reproductives were sexed, weighed and immediately placed in experimental colonies.

We established four groups of colonies. Colonies in two groups contained one pair of primary reproductives, whereas colonies in the remaining groups contained a pair of neotenic reproductives and three third- or fourth-instar larvae. Larvae were added to the latter colonies to ensure that reproductive maturation would occur, because isolated neotenics delay reproductive development (Greenberg et al. 1978, Greenberg and Stuart 1979, Brent and Traniello 2001). Although the presence of three independent larvae is sufficient to promote the maturation of neotenics (Brent and Traniello 2001), the small number and size of the larvae limits their impact on the food supply, which could negatively influence female development (Lenz 1994). Primary reproductives were paired randomly with respect to colony of origin, but paired neotenics were always siblings to simulate the normal conditions under which replacement reproductives develop. All colonies nested in covered 67 ml plastic cups (Solo Cup, Chicago, IL) containing 2 g (dry weight) of birch sawdust, and were maintained at 23°C and a photoperiod of 14:10 (L:D) h. The sawdust for one group of primaries ($n = 74$) and neotenics ($n = 95$) was moistened with 6 ml of distilled water, and the sawdust for the remaining groups of primaries ($n = 86$) and neotenics ($n = 74$) was moistened with 6 ml of a 0.05 μl solution of uric acid in distilled water (99% pure, Sigma, St. Louis, MO). The sawdust was thoroughly mixed as the uric acid solution was added to ensure an even distribution, and was then hand pressed to drain off excess water.

The nitrogen contents of the two food sources were determined using a Thermoquest NC2500 Elemental Analyzer (CE Instruments, Milan, Italy), which provides an estimate of nitrogen content that is accurate to within 0.003%. Eight samples were analyzed from each food source. Untreated birchwood contained an average of 0.189 ± 0.002% nitrogen; uric acid-supplemented birchwood contained 0.241 ± 0.003% nitrogen.

The growth and ovarian maturation of neotenic females were recorded at 0, 30 and 60 d after colony establishment. The fresh live body mass of each neotenic was recorded on a Mettler AE-163 (Greiffensee, Switzerland) balance before and after each experiment. Before dissection, termites were preserved in Dietrich's fixative (Gray 1954) for a minimum of two weeks. Dissections were carried out using a Wild M5A dissecting scope. Incisions were made along both pleural intersegmental membranes, between the second and third sternites, and the ovaries were removed for microscopic examination. An Olympus BH-2 stereoscopic microscope, (Tokyo, Japan), was used to determine the total number of ovarioles in each ovary. The length and width of the terminal oocytes in the first five ovarioles of each ovary were measured using a stereoscopic microscope fitted with an ocular micrometer. Oocytes were assumed to be roughly cylindrical; therefore, oocyte volume was estimated as $\pi \times [\text{radius}]^2 \times [\text{length}]$. Oocytes were categorized as vitellogenic if their volume was at least 0.01 mm^3 (Hewitt et al. 1972). Three measures of female growth and ovarian maturation were recorded: (1) percent change in body mass; (2) average number of functional ovarioles; and (3) fecundity, estimated from the cumulative number of eggs, newly eclosed larvae and vitellogenic terminal oocytes produced before sampling. The average mass of an egg was measured by pooling multiple oocytes to estimate the percent of total body mass lost through oviposition.

All statistical analyses were performed using SigmaStat (SPSS 1997). Significant interaction effects between the number of days before sampling and the treatment coupled with consistent violations of normality necessitated using the nonparametric Mann-Whitney rank sum test (Sokal and Rohlf 1995) to compare percent change in body mass, average ovariole number and fecundity between treatment groups on individual days. Probability values ($\alpha = 0.05$) were adjusted for multiple tests using Scheffé correction (Miller 1981).

Results

Supplementing dietary nitrogen modified the growth and ovarian maturation of both primary and neotenic reproductives, although the effects on ovariole number and fecundity were quite different in each reproductive form. Primary females allowed to feed on untreated wood for 60 d increased their body mass by an average (±SE) of 70.52 ± 2.55%, whereas
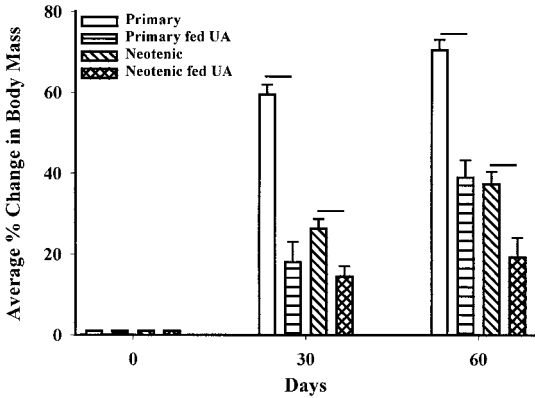
Fig. 1. Average percent increase in body mass ($\pm$SEM) of primary and neotenic females fed untreated wood, or wood treated with a 0.05% solution of uric acid. Horizontal lines above bars indicate comparisons between treatments that were significantly different ($P < 0.05$) for each reproductive form.

Fig. 3. Average fecundity ($\pm$SEM) of primary and neotenic females fed untreated wood, or wood treated with a 0.05% solution of uric acid. Horizontal lines above bars indicate comparisons between treatments that were significantly different ($P < 0.05$) for each reproductive form.

those provided nitrogen-enriched wood increased body mass by $38.94 \pm 4.31\%$ (Fig. 1; Mann-Whitney, $T = 595.0$; df $= 20, 21$; $P < 0.001$). A similar significant difference was observed in neotenics; those fed untreated wood for 60 d gained $37.26 \pm 3.08\%$ more mass, while those supplied with uric acid gained $19.24 \pm 4.85\%$ (Mann-Whitney, $T = 383.0$; df $= 17, 17$; $P = 0.003$).

The addition of uric acid influenced the number of functional ovarioles, but primary and neotenic females had different responses (Fig. 2). Primaries fed untreated or treated wood had similar numbers of functional ovarioles after 60 d, with respective averages of 34.73 ± 0.78 and 32.81 ± 0.98 ovarioles per ovary (Mann-Whitney, $T = 481.0$; df $= 20, 21$; $P = 0.115$). Neotenic females, however, developed significantly

more ovarioles when fed uric acid, averaging 35.21 ± 1.09 per ovary on day 60, compared with females fed untreated wood (31.85 ± 1.22 ovarioles; Mann-Whitney, $T = 235.0$; df $= 17, 17$; $P = 0.033$).

The addition of nitrogen supplementation affected egg production in primaries and neotenics in a pattern similar to that seen with ovariole number (Fig. 3). The fecundities of primary females fed nitrogen-supplemented and unsupplemented diets did not differ. Those eating untreated wood produced a mean of 7.75 ± 0.70 eggs by day 60, and those given supplemental uric acid produced 7.19 ± 0.81 eggs (Mann-Whitney, $T = 444.5$; df $= 20, 21$; $P = 0.531$). But the addition of uric acid significantly increased the number of vitellogenic eggs in neotenics from $2.47 \pm 0.70 - 6.59 \pm 1.50$ (Mann-Whitney, $T = 205.0$; df $= 17, 17$; $P = 0.002$). The average mass of an egg was 3.07×10^{-4} g ($n = 59$).

Discussion

Enhancing the diet of maturing reproductives with exogenous nitrogen appeared to benefit only neotenic females. Primary reproductives fed a uric acid-supplemented diet gained less body mass after 60 d and developed similar numbers of functional ovarioles and oocytes compared with females fed untreated wood. Neotenics provided with a similarly supplemented diet also gained less body mass, but produced significantly more functional ovarioles and oocytes. One key factor that may have influenced how reproductives responded to uric acid was the developmental origin of each form. Primary reproductives are derived from older termites that have gone through nymph and alate stages within their natal nest before their dispersal flight (Miller 1969). Their well-developed fat bodies may store resources sufficient to ensure that they can found a colony with only the assistance of their mate (Grassé 1949, Nutting 1969). Metabolized flight muscles could also serve as an additional source
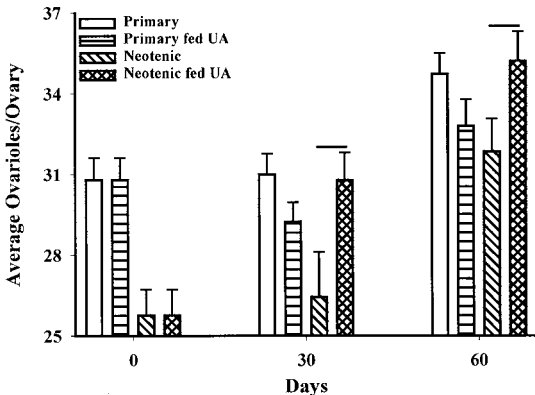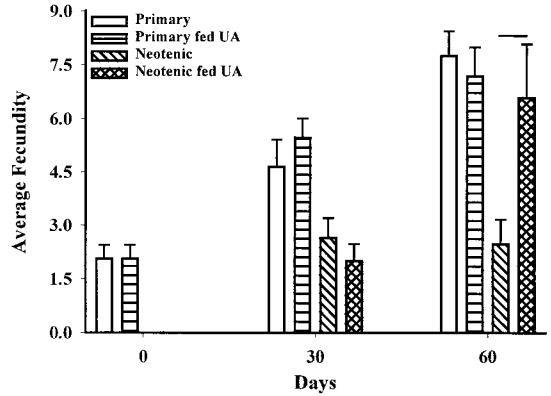

Fig. 2. Average number of functional ovarioles per ovary ($\pm$SEM) of primary and neotenic females fed untreated wood, or wood treated with a 0.05% solution of uric acid. Horizontal lines above bars indicate comparisons between treatments that were significantly different ($P < 0.05$) for each reproductive form.

of energy and nitrogen during the colony's incipient stage (Grassé 1949). Neotenic reproductives, however, transform into reproductives from larvae in a single molt (Castle 1934, Miller 1969), and the neotenics we used in our study were significantly smaller than primaries. Newly molted neotenics probably start with the uric acid reserves similar to those of larvae. Shellman-Reeve (1990) found that larvae of Z. *nevadensis* had 2% of the average uric acid level of alates. Although some species can derive nitrogen from the digestion of dead intestinal symbionts (Honigberg 1970, Collins 1983) and the fixation of atmospheric nitrogen by endosymbiotic bacteria (Benemann 1973, Breznak et al. 1973, Prestwich et al. 1980, Collins 1983, Bentley 1984, Higashi et al. 1992, Ohkuma et al. 1999), *Zootermopsis* derives only a small portion of their nitrogen budget from these sources (Benemann 1973, Breznak et al. 1973). Therefore, the low level of endogenous nitrogen reserves likely disposes the reproductive development of newly molted neotenics to be highly responsive to exogenous sources of nutrition.

A second factor that may have influenced the ability of reproductives to benefit physiologically from nitrogen supplementation was the presence of larval helpers. We tried to emulate the typical biological conditions under which both primary and neotenic females complete their reproductive maturation: paired primaries were left in isolated colonies while paired neotenics were allowed to nest with larvae. Neotenics nesting with larvae complete their reproductive maturation significantly faster than those in isolated pairs (Greenberg et al. 1978, Brent and Traniello 2001), suggesting that nonreproductive nestmates help neotenics to quickly acquire the resources necessary for their reproductive maturation. Consumption and digestion rates constrain how quickly termites can accumulate nutrients from wood (Slansky and Scriber 1985). Many termites engage in trophallaxis, providing nestmates with secretions containing nitrogen and other products of digestion (McMahan 1969, Breznak 1982, Shellman-Reeve 1990, Machida et al. 2001). Nutritive assistance would help circumvent the dietary limitations on a neotenic's maturation and fecundity. Trophallactic secretions provided by larvae feeding on uric acid-supplemented diets may have amplified the delivery rate of nitrogen to the neotenics so that they could rapidly produce the amino acids necessary for reproductive development and oogenesis.

Primary females, however, did not nest with larvae and therefore had no assistance with brood care or resource acquisition, other than what the male could provide (Shellman-Reeve 1990, Rosengaus and Traniello 1991). Although primary females given supplemental nitrogen should have had sufficient resources to produce additional eggs, the primary pairs may not have been capable of caring for larger broods. Oogenesis, long-term brood care and colony maintenance can be energetically costly, often leading to weight loss in reproductives of many lower termites (Shellman-Reeve 1990, Nalepa and Jones 1991). Attempting to care for a larger than average first brood

without assistance from nutritionally independent larvae might put the pair of primaries at risk, or could decrease the individual probability of survival for their offspring. We have observed that primaries nesting under similar conditions with untreated wood but with larvae exhibited significant increases in fecundity (Brent and Traniello 2001). Although the increase may have been the result of the larvae providing supplemental nitrogen, the present results indicate that the positive effect of larvae on fecundity was likely due to the primaries being released from having to care for the brood.

The reduced mass gain observed in primary and secondary reproductives fed wood treated with uric acid may have several causes. By day 60, neotenic females fed uric acid-treated wood laid more eggs than those fed untreated wood, accounting for some mass loss. However, the weight of an individual egg is quite small, so that differences in oviposition rates account for only a small fraction of the mass difference in neotenics and fail to account for similar differences in primary female mass gain. A more likely source of the differences in mass gain for both primaries and neotenics may have been a change in the consumption rate of food as a result of the enhanced diet. One possibility is that the uric acid acted as a feeding deterrent by rendering the wood less palatable. However, uric acid at the concentration used in this experiment is nontoxic to termites (Breznak 1982). Moreover, a uric acid concentration of 0.15% did not deter Z. *nevadensis* primaries from feeding (Shellman-Reeve 1990).

Alternatively, the increased nutritional quality of the uric acid-treated wood may have enabled both primary and neotenic females to reduce their consumption rate while still meeting their dietary needs. Termites may be ungulate-like in that they ingest large quantities of low-quality food to meet their dietary needs (Montgomery and Baumgardt 1965, Robbins 1993, Van Soest 1994), and their consumption rate may vary according to the quality of their food. Food intake is normally distributed as a function of its nutritive value in many ungulates, so that less food is ingested when it is either very low or high in quality and the largest amounts are consumed when the food is of intermediate nutritional value (Robbins 1993). Numerous insect species are known to vary their consumption rate in response to differences in diet quality (reviewed in Slansky and Scriber 1985), and termites have also exhibited such flexibility (Shellman-Reeve 1990, Machida et al. 2001, reviewed in Lenz 1994). Primary reproductives of Z. *nevadensis* increased their feeding rate when the nitrogen content of their diet was increased from 0.03 to 0.18% (Shellman-Reeve 1990), which approximates a shift from a very low to an intermediate quality food. In our study, termites were fed wood of intermediate and high quality, with 0.19% and 0.24% average nitrogen, respectively. The addition of uric acid may have increased the nitrogen content of the food, allowing reproductives to ingest considerably less wood to meet their dietary needs, thereby reducing their gut contents and body mass.

Machida et al. (2001) found similar reductions in the frequency of trophallactic exchanges between nestmates with increased nitrogen availability. Confirmation of this theory will require measures of the consumption rate and gut contents for reproductives fed food of different quality.

The enhanced diet may have also led to a reduction in uric acid storage. Stored uric acid can comprise a significant portion of the dry mass of termites (Potrikus and Breznak 1981). Storing less uric acid in the fat body may result in substantial weight reduction. A mechanism that regulates uric acid storage would be beneficial if there were a metabolic cost associated with uric acid retention; however, changes in dietary nitrogen have not been conclusively shown to influence the rate of nitrogen fixation (Curtis and Waller 1997). *Z. angusticollis* primaries nesting with larvae show a similar decline in the rate of mass gain (Brent and Traniello 2001). Reproductives may not sequester as much nitrogen if it is easily acquired from the environment or nestmates, or they may sequester more if their nitrogen requirements increase or the external concentration decreases. Such a conditional response would help to ensure females of a constant supply of nitrogen adequate to their needs.

In summary, supplemental nitrogen did not affect the fecundity of primary females but did promote reproductive development in neotenics. The high metabolic cost of the long-term care for her first brood probably constrains the reproductive output of a young primary queen more than nitrogen availability. It is likely that a primary female becomes more responsive to exogenous nitrogen as she ages, given that nitrogen availability appears to strongly influence reproductive output in older colonies of the closely related *Z. nevadensis* (Shellman-Reeve 1994). The presence of larval helpers during the later stages of colony development may enable primary females to more fully use nitrogen resources in increasing her fecundity. Neotenics, however, initiate their reproduction with few resource reserves, so that their maturation and fecundity are more dependent on exogenous nitrogen. Further study is needed to determine how nitrogen availability may influence the fecundity of reproductive females during different stages of their life history.

Acknowledgments

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