Micronutrient Changes in Colonies of the Ant Temnothorax curvispinosus (Hymenoptera: Formicidae) During the Colony Cycle

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ABSTRACT To gain a better understanding of micronutrient distribution, and how this relates to growth and survival of a social insect colony, this study focused on micronutrient levels within colonies of the ant Temnothorax curvispinosus Mayr during four periods of the colony cycle—1) Pre-Reproductive, Reproductive, Pre-Winter, and Winter. Ten colonies were collected from the field monthly and were analyzed for levels of Ca, Cu, Fe, K, Mg, Mn, Na, Ni, and Zn. Several overall trends were noted. 1) A general loss of some micronutrients within the workers and queens, and the colony as a whole in the Winter Period. 2) Levels of Mg and Mn increased during the Pre-Reproductive and Reproductive Periods in workers and queens while levels of Ca and Zn only increase in the queens during these periods. 3) Levels of K peaked in the Pre-Winter Period in workers and queens while levels of Na only increased in workers during this period. 4) Levels of Mn were lower in alates than the workers, queens, or brood during the reproductive period. The potential reasons for the observed patterns are discussed.

KEY WORDS micronutrient, ant, Temnothorax, nutrition, caste

Temperate-zone organisms experience large fluctuations in the availability of resources across seasons. During the winter, when resource availability is minimal, these organisms display a variety of survival strategies. Within the social Hymenoptera, annual colonies such as Polistes or Bombus transition from an ergonometric (growth) period to a reproductive period (Wilson 1971, Hunt 2007). The final output of the colony is overwintering reproductives that initiate new colonies the following year. The change from ergonometric to reproductive periods is accompanied by a shift in foraging behavior (Jeanne and Taylor 2009) and a shift in resource distribution (Judd et al. 2010). Perennial colonies also have ergonomic and reproductive periods (Oster and Wilson 1978, Hunt 2007), but these periods are repeated over several years. Thus, perennial colonies in temperate climates must divide their resources between reproduction, growth, and storage in order for entire colonies to survive to the following year (Judd 2011).

Of the perennial social Hymenoptera, ants have been extremely successful in adapting to temperate climates (Hölldobler and Wilson 1990) and have developed several methods to store food. Most ants store resources in their bodies, but some species cache food in addition to their internal resources (Judd 2011). In some cases, certain individuals act as repletes (Wilson 1974, Tschinkel 1993b, Yang 2006). Because ants use their bodies as storage vessels, their nutrient content should change depending on where the colony is in its annual cycle. When a colony is reproducing, workers should gather and store nutrients that are necessary for growth and pass these nutrients onto the queen and brood, which are the individuals that reproduce and grow, respectively. As winter approaches, ants should switch to storing nutrients necessary for overwintering. Ant colonies change the intake and distribution of macronutrients during the year between the reproduction or growth periods of the colony cycle and those periods before and during overwintering. The intake and storage of proteins and amino acids increases when colonies are in a reproductive or growth phase whereas carbohydrates and lipids are the main macronutrients gathered and stored before winter. (Stein et al. 1990; Cannon and Fell 2002; Judd 2005, 2006; Cook et al. 2010). These changes reflect the different nutritional needs of the different “life stages” (defined here as including all adult castes and all developmental stages, see Judd et al. 2010). In addition, macronutrients are distributed through a colony based on nutritional needs for the different life stages. Proteins are distributed to the life stages that are growing (larvae) or reproducing (queens) (Vinson 1968; Tschinkel 1993a; Cassill and Tschinkel 1995, 1999; Weeks et al. 2004; Loke and Lee 2006; Cook et al. 2010).

Unlike macronutrients, the fluctuation and distribution of micronutrients in social insect colonies is not well documented (Judd and Fasnacht 2007). Micronutrients include metal cations and their nonmetal...
conjugate anions and are used in such diverse functions as cell regulation, gas transfer, protein structure, and enzyme function (da Silva and Williams 2001). The specific functions of micronutrients in insects is still poorly understood (Cohen 2004). However, studies have indicated that micronutrients are important for a number of physiological functions including growth, fecundity, and food storage (summarized in Table 1). Thus, we hypothesize that like macronutrients, micronutrients should be 1) distributed according to the relative needs of each life stage in an ant colony, and that 2) the intake of these nutrients should change according to phase of the colony.

It is likely that micronutrients in perennial colonies will not only differ between different life stages but may fluctuate within a life stage during the colony cycle, just as levels of macromolecules fluctuate. A few studies have shown that micronutrients are not evenly distributed within a hymenopteran colony. For example, manganese levels were found to be higher in adult foragers of wasps (Bowan 1950, Judd et al. 2010), bees (Ben-Shahar et al. 2004), and ants (Levy et al. 1974, 1979; Judd and Fasnacht 2007). Manganese has been associated with sugar detection in bees and fruit flies and may be involved in stimulating foraging (Orgad et al. 1998, Ben-Shahar et al. 2004). Studies comparing relative nutrient levels in worker and larva ants have been less consistent. Judd and Fasnacht (2007) found that in the ant Myrmica punctiventris Roger the larvae ended up with the majority of the micronutrients with the exception of manganese. However; Solenopsis workers were found to contain higher levels of several micronutrients than larvae (Levy et al. 1974, 1979). All of the proceeding examples suggest that not all micronutrients follow the same distribution pattern within a colony. What has yet to be determined is whether there is a clear distinction between the acquisition and distribution of micronutrients during the growth or reproductive periods and storage or overwintering periods of a perennial colony.

The model organism we used to examine the changes in micronutrient distribution is the ant Temnothorax curvispinosus Mayr. T. curvispinosus colonies inhabit small hollow structures such as acorns, hickory nuts, and hollow sticks (Talbot 1957, Herbers and Stuart 1996). Thus, the entire colonies can be easily sampled, allowing us to examine levels of micronutrients within life stages of the entire colony. The colony cycle of T. curvispinosus can be divided into four biologically relevant periods (Fig. 1): Prewinter (September–November) in which colonies are foraging, primarily to prepare for winter; Winter (December–February) colonies overwinter and must rely on internal stores; Pre-Reproductive (March–May) colonies emerge from overwintering and forage to prepare for reproduction; and Reproductive (June–August) colonies are caring for larvae and alates (adult reproductives). In this study, we examined the change in levels of nutritive cations calcium (Ca), copper (Cu), iron (Fe), potassium (K), magnesium (Mg), manganese (Mn), sodium (Na), nickel (Ni), and zinc (Zn). Colonies were sampled over an entire year to determine the fluctuations and distribution of these micronutrients within individual life stages and the colony as a whole to gain insight into the seasonal dynamics of the micronutrients in an ant colony. Based on historical literature (Table 1), we predicted that nutrients affecting seasonal physiological functions such as fecundity, growth, food storage, and foraging behavior should show more differences between castes and the four periods than those nutrients involved in constant physiological functions (e.g., immune function, cuticular strengthening, and general metabolism). In addition, because the functions of these nutrients is still not well understood, we can also gain further clues of other potential functions of these micronutrients.

| Nutrient | Function | Organism | References |
|----------|----------|----------|------------|
| Ca       | Increase fecundity | *Acheta domesticus* L. | McFarlane 1991 |
|          | Ovarian development | *Helothis ireneus* F. | Pszczolowski et al. 2008 |
|          | Ice nucleation | *Eurosta solidaginis* Fitch | Mignano et al. 1996 |
| Cu       | Cuticular hardness | *Incisitermes minor* Hagen & *Cryptotermes domesticus* Haviland | Ohmura et al. 2007 |
|          | Cuticular pigmentation, growth | *Acheta domesticus* L. | McFarlane 1974, 1976 |
|          | Egg coat | Many insects | Melvin 1931 |
| Fe       | Protein synthesis and metabolism | All insects | Nichol et al. 2002 |
| K        | Increase level of lipids in fat body | *Bombus mori* L. | Bhattacharya and Kalival 2005a,b |
|          | Increase fecundity and growth | *Acheta domesticus* L. | McFarlane 1991 |
|          | Urate transport into fat body | *Hyalophora cecropia* L. | Jungreis and Harvey 1975 |
| Mg       | Aids in ecdysis | *Hyalophora cecropia* L. | Jungreis and Tojo 1973 |
|          | Glycolysis | All insects | Murphy and Wyatt 1965 |
| Mn       | Increase fat body content | *Bombus mori* L. | Bhattacharya and Kalival 2005b |
| Na       | Affects sugar sensitivity | *Drosophila melanogaster* Meigen | Orgad et al. 1998 |
|          | Cyticlar hardness | *Hymenoptera* | Quicke et al. 1998 |
|          | Ovarian development | *Acheta domesticus* L. | McFarlane 1991 |
| Ni       | Increase fecundity and growth | *Tymelicus lineola* Ochsenheimer | Pivnick and McNeil 1987 |
|          | Increase fecundity | *Hyalophora cecropia* L. | Zachariassen et al. 2004 |
|          | Freeze tolerance | *Many species* | Islam et al. 2004 |
| Zn       | Growth | *Bombus mori* L. | Quicke et al. 1998, Schofield et al. 2002, Ohmura et al. 2007 |
|         | Cyticlar hardness | *Hymenoptera, Isoptera* | (Willott and Tran 2002) |
|          | Immune function | *Mandra sexta* L. | (Willott and Tran 2002) |
The ant specimens were dried at 35°C. For each colony, the average level of each nutrient per unit mass was determined for each life stage (workers, queens, brood, and alates). Levels of nutrients per unit mass were also determined for each colony. The data were log transformed before the subsequent analyses. Because the data were log transformed, the geometric means and 95% CIs were back transformed and are depicted in Figs. 2 and 3. Hereafter, “level” of a nutrient refers to level per unit mass.

**Materials and Methods**

**Sampling.** *T. curvispinosus* colonies were collected from September 2004 to August 2005 at Juden Creek Wildlife Management Area (37° 20’ N, 89° 30’ W). Approximately 10 colonies were collected each month, on or around the 15th of that month. Owing to the fact that *T. curvispinosus* regularly establish satellite colonies (Herbers and Stuart 1996), no ≥3 colonies were selected from within a 10-meter radius each month, and a different area was sampled each month. Colonies were transported back to the laboratory in separate sealable plastic bags. Within 72 h of removing the colonies from the collection site, the colonies were separated from their nest matrix and stored at −20°C.

Colonies were separated by life stage (workers, queens, gynes, males, and brood) into 1.8-ml scintillation vials. All of the specimen vials were washed with trace metal grade HCl. Then triple rinsed with deionized water before use. One to 10 workers were placed in individual vials. Each queen and alate (gyne or male) was placed in individual vials. The ant specimens were dried at 35°C until a constant mass was reached and then weighed to the nearest 10 μg.

**Digestion and Analysis.** The samples were analyzed using Inductively Coupled Plasma–Optical Emission Spectrometry (ICP–OES). Before analysis, the samples were washed by using 0.1 ml of concentrated nitric acid. After the addition of the acid, samples were dried on a hotplate. After cooling, the samples were reconstructed with 1 ml of 5% nitric acid, spiked with Scandium (Sc) to a concentration of 1 ppm. This element was used as an internal standard. Sc is uncommon in living systems, and is sensitive and stable when analyzed for content by ICP–OES (Skoog et al. 2006). When analyzed, emission for each element was detected at the following wavelengths: Ca, 317.933 nm; Cu, 324.754 nm; Fe, 258.204 nm; K, 766.491 nm; Mg, 279.079 nm; Mn, 257.610 nm; Na, 589.592 nm; Sc, 361.384 nm; and Zn, 213.856 nm.

After analysis, all samples were then normalized by dividing the emission of the unknown, by the emission of the internal standard, Sc. Standards used included—0.10, 0.50, 1.0, 3.0, 5.0, and 10.0 ppm for all of the elements analyzed. Controls (blanks) were also analyzed and were prepared in the same manner as the unknowns. The values obtained from the controls were then subtracted from the unknowns for all of the elements except Na. Most of the controls became contaminated with Na, and so were not used to correct the unknowns for that element. The amount of variation of Na levels in the controls that were not contaminated were extremely small (<0.00035%); thus, using uncorrected data for Na would not produce artificial effects within the analyses.

**Data Analysis.** The amount of nutrient per gram of dry mass was determined for each sample. For each colony, the average level of each nutrient per unit mass was determined for each life stage (workers, queens, brood, and alates). Levels of nutrients per unit mass were also determined for each colony. The data were log transformed before the subsequent analyses. Because the data were log transformed, the geometric mean and 95% CIs were back transformed and are depicted in Figs. 2 and 3. Hereafter, “level” of a nutrient refers to level per unit mass.

**Colony-Level Analysis.** A MANOVA (PROC GLM using SAS/STAT Software, SAS Institute Inc., Cary, NC) comparing period (independent variable) with the levels of each nutrient (dependent variables) was performed followed by a Tukey Honest Significance Difference Test (THSD).

**Life Stage-Level Analysis.** This study was testing whether there were differences between life stages within a period and whether there were changes within a life stage between periods. However, the brood and alates were only present during the reproductive period. One solution to this problem was to create a single independent categorical variable that represented a life stage within a period hereafter called LSPD (Life Stage-Period). A similar analysis to the colony-level MANOVA was performed but LSPD was used as the independent variable instead of period.

**Results**

Changes at the Colony Level Between Periods. The overall ANOVA for the colony-level analysis was significant (λ = 5.75; DF₉ = 27; DFᵣ = 234.28; P < 0.0001). The only elements that showed significant changes between the four periods at the colony level.
were K, Mg, and Mn (Table 2). Levels of K and Mn were significantly lower in the Winter Period than the other three periods \( (P_K < 0.05; P_{Mn} < 0.0005, \text{THSD; Fig. 2D and F}) \). Levels of Mg were lower in the Winter Period than the Pre-Reproductive and Reproductive Periods \( (P < 0.0001, \text{THSD; Fig. 2E}) \). These results suggest that levels of three micronutrients were reduced before overwintering and regained during the Pre-Reproductive phase.

Comparisons Between LSPDs. The overall ANOVA for the LSPD analysis was significant \( (\lambda = 3.97; DF_N = 81; DF_D = 1062.4; P < 0.0001) \). There were no significant differences between the LSPDs for Cu and Fe (Fig. 3B and C; Table 2). Although significant
Comparisons Within Workers and Queens Across the Periods. Of the nine micronutrients examined, only K, Mg, Mn, and Na showed significant changes in levels per unit mass in workers (Fig. 3D–G). Levels of K dropped significantly in workers from the Pre-Winter to the Pre-Reproductive period. Differences between LSPDs in the model were found for Ni (Table 2), the THSD analysis revealed no biologically relevant differences (Fig. 3H). All other elements showed biologically significant results (Table 2).
Table 2. Results of the MANOVA for levels of micronutrients in the LSPDs of *T. curvispinosus*

| Nutrient | F    | p    |
|----------|------|------|
| Ca       | 3.07 | 0.002|
| Cu       | 1.03 | 0.42 |
| Fe       | 1.82 | 0.067|
| K        | 3.82 | 0.0002|
| Mg       | 8.76 | <0.0001|
| Mn       | 8.09 | <0.0001|
| Na       | 3.03 | 0.0022|
| Ni       | 3.28 | 0.001|
| Zn       | 4.61 | <0.0001|

Period to the Winter Period (*P* = 0.015, THSD; Fig. 3D). Levels of Mg in workers were significantly higher in the Reproductive Period than the Winter Period (*P* = 0.023, THSD; Fig. 3E). These results suggest that the workers were increasing their levels of Mg in the Pre-Reproductive and Reproductive periods. There was a significant increase in levels of Mn from the Winter Period to the Pre-Reproductive Period (*P* = 0.0124, THSD; Fig. 3F). Unlike Mg, the levels of Mn in workers were not significantly higher in the Reproductive Period than the Pre-Winter Period (*P* = 0.011, THSD; Fig. 3G). Thus, the levels of Na in workers appeared to have gradually dropped from the Pre-Winter Period to the Reproductive Period.

Queens showed significant changes in Ca, K, Mg, Mn, and Zn (Fig. 3). The levels of K in queens followed the same pattern as found in workers; i.e., levels were lower in the Winter Period than the Pre-Winter Period (*P* = 0.0078, THSD; Fig. 3D). Levels of both Ca and Mn in the Winter Period were significantly lower than the levels in the Reproductive Period (*P* < 0.011, *P* < 0.0001, THSD; Fig. 3A and F). Similarly, levels of both Mg and Zn were significantly lower in the Winter Period than the other three periods (*P* < 0.0001, THSD; Fig. 3E and I). Comparison of Micronutrient Content Between Life Stages. There were only a few differences between life stages within a period. Queens had significantly lower levels of Ca (*P* = 0.0003, THSD; Fig. 3A) and Mg (*P* = 0.0013, THSD; Fig. 3E) and Zn (*P* < 0.0001, THSD; Fig. 3I) than workers in the Winter Period. There were no other significant differences between workers and queens within the other three periods. During the Reproductive Period, queens, workers, and brood had significantly higher levels of Mn than alates (*P* < 0.0001, *P* = 0.0001, *P* = 0.032, THSD; Fig. 3F). There were no other significant differences between the life stages during this period.

### Discussion

The results suggest that some of the micronutrients fluctuated during the colony cycle at the colony level and within life stages. We predicted that nutrients involved in seasonal physiological function such as growth, storage, reproduction, and foraging (Table 1) should have fluctuated between the four periods. In general, apart from Zn and Ni, the results did match the predictions. K, Mg, and Mn all showed significant fluctuations at the colony level and within life stages, and Ca, Zn, and Na fluctuated within and between life stages, but not at the colony level. For most of these elements, the main pattern was a reduction of nutrient levels during the Winter Period. At the colony level, K, Mn, and Mg were at their lowest levels during the Winter Period. These micronutrients were at their lowest levels in both workers and queens, which probably explains the colony level drop. Thus, the colony level pattern was driven by similarities in the workers and queens. Some of the elements (Cu, Fe, and Ni) never varied between periods at the individual life stage level or colony level. Ni was found to only be needed in small quantities in *Bombyx mori* L. (Islam et al. 2004) so the lack of change in Ni levels in *T. curvispinosus* may reflect this. Cu and Fe were predicted not to change based on what is known about their functions (Table 1).

Although levels of Mg and K were the same for workers and queens across the four periods, levels of Mg increased more rapidly, peaking in the Reproductive Period compared with the levels of K, which peaked in the Pre-Winter Period. Mg is important for glycolysis and trehalose synthesis (Murphy and Wyatt 1965), which would be important for all individuals when the colony is active. During the Winter Period, the colonies are in diapause; thus, the reduction in Mg may be part of a mechanism that slows down the ants’ metabolism. Interestingly, the low levels of Mg in *T. curvispinosus* contrasts the pattern in overwintering gynes of *Polistes metricus* Say, which had high levels of Mg (Judd et al. 2010). The gynes initiate reproduction fairly soon after emergence so they may need to retain enough Mg for this purpose. *T. curvispinosus* colonies wait for several months before initiating reproduction, thus, could afford to lose Mg over the winter.

In the Pre-Winter Period, levels of K and Na were at their highest levels. This is when colonies increase their carbohydrate and lipid stores (Ricks and Vinson 1972, Judd 2006), which have been associated with elevated levels of K (Table 1). Ricks and Vinson (1972) found that glycerol levels in *Solenopsis saevisima rishteri* Forel are highest during the fall months (September and November), which would correspond to the Pre-Winter Period in this study. Glycerol plays an important role in freeze tolerance in insects (Lee et al. 1998, Lee and Costanzo 1998). High levels of Na and K are also important for freeze tolerance (Lee and Costanzo 1998, Zachariassen et al. 2004); thus, an increase in levels of these molecules during the Pre-Winter Period would probably help the colony prepare for overwintering. Unlike K, the overall colony Na levels did not change nor did the levels in the queen. Na has been found to be important for the transport for amino acids through the insect gut wall (McLean and Caveney 1993, Wollersberger 2000). Workers are sterile and do not grow which in turn reduces their need for amino acids. Thus, one possible explanation for the reduction of Na in workers but not
the queens is that the workers passed their Na to other life stages during the Reproductive Period. Larvae are growing and need to absorb amino acids and queens would need to absorb amino acids for egg production which would improve fecundity. Queens would also need Na for overwintering which could explain why Na levels did not change significantly in queens.

Workers and queens did not show a similar pattern for changes in levels of Mn. Levels of Mn peaked in the workers during the Pre-Reproductive period and peaked in the queens during the Reproductive Period. As mentioned earlier, Ben-Shahar et al. (2004) found that the activity of the gene malvolio, a Mn transporter, increases in foraging honey bees. If this mechanism is similar in ants, then the low levels of Mn in workers during the Winter Period could reduce the drive to forage. Once the colony enters the Pre-Reproductive Period, the colony needs to build nutrient stores to prepare for brood care. The rise in Mn in the workers during the Pre-Reproductive Period may be tied to the increase in foraging at this time. The fact that queens’ levels of Mn peaked later suggests that Mn may be important in reproduction. Mn has been found to be important for reproduction in vertebrates (Hill and Mathers 1968, Mathers and Hill 1968, Takeuchi et al. 1981, Watanabe et al. 1997, Maage et al. 2000) but has not been looked at in this regard in invertebrates. It is possible that Mn serves different functions in queens then workers, and this difference could explain the delayed increase in the levels of Mn in the queens relative to the workers. Mn was also the only micronutrient that showed any difference between the alates and other life stages. While in the colony, alates are not involved in foraging or reproduction. Therefore, the need for nutrients involved in these functions would not be necessary for alates. Relatively low levels of Mn have also been reported in emerging adults of Vespuila, Vespa, and Polistes (Bowen 1950, Judd et al. 2010). Low levels of Mn in emerging alates may be a trend in social Hymenoptera.

Levels of both Ca and Zn only fluctuated in the queens. Ca is important for mitotic division and was shown to be important for the fecundity of the cricket Acheta domestica L. (McFarlane 1991) and the moth Heliothis virescens F. (Pszczolowski et al. 2008). Zn was reported to be essential for ovarian development in mice (Tian and Diaz 2012). The possible role of Zn in ovarian function in insects has not been studied. Ovary function would be at its lowest in the Winter Period, which could explain the drop in these two elements in queens. Workers are sterile which could help explain the constant levels in workers.

One interesting result in this study was that the different micronutrients did not rise at the same time. For example, levels of Mn and Mg peaked much earlier than levels of K and Na. This difference suggests the possibility that the ants could change their feeding preferences based on which micronutrients are required in the colony in addition to the colony’s micronutrient needs. Indeed, low levels of macronutrients in workers have been shown to induce foraging in ants (Blanchard et al. 2000, Judd 2005). Micronutrients were found to affect the foraging behavior of Lepidoptera (Arns et al. 1974, Smedley and Eisner 1995, Mollenman 2009), Isoptera (Botch et al. 2010, Botch and Judd 2011), Orthoptera (Simpson et al. 2006, Shen et al. 2009), and Halictidae (Barrows 1974). Vinson (1970) demonstrated that Solenopsis saecissima risheri foragers were able to detect and respond to many elements including the ones studied here. Some nutrients such as Zn and Mg were preferred over others (e.g., K). The exact time these colonies were collected was not specified, but the author did mention the colonies contained brood, suggesting the colonies were in a Reproductive Period. The micronutrient preference of S. saecissima risheri would correspond to the nutrient levels found in T. curvispinosus colonies during this period. In a field study, Kaspari et al. (2008) found that ants in environments that have low Na abundance will forage at NaCl baits. Therefore, it is possible that in the Pre-Winter Period, T. curvispinosus may increase their preference for Na and K, especially when their Na levels are at their lowest levels. Whether or not other micronutrients influence ant foraging behavior remains to be seen.

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