Altitudinal variation in ant–aphid mutualism in nitrogen transfer of oak (*Quercus liaotungensis*)

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Abstract Ant–plant relationship is a model for the study of the ecology and evolution of interspecific interactions. In direct ant–plant mutualism (i.e., plants providing food or nesting places for ants, and ants protecting the plants in return) ants provide nutrients to plants. However, whether a similar mechanism exists in indirect ant–plant mutualism (i.e., an ant–aphid–plant system) remains unknown. In this work, we used the ¹⁵N stable isotope method to study altitudinal variations in the roles of ants in the nutrient transfer of oak (*Quercus liaotungensis*). Our work shows that ants deliver nitrogen in indirect ant–plant interactions, and that the effect of nutrient transfer differed significantly with altitude. Ants’ trophic level at high altitudes was significantly lower than that at low altitudes, indicating that the degree of ant–aphid mutualism was greater at high altitudes, which may be beneficial in nitrogen transfer. Our work suggests that ant–aphid mutualism might be context dependent, such that it affects nutrient transfer in the food web, and that this context dependency is an important factor that influences altitudinal variation in nutrient transfer.

Keywords ¹⁵N stable isotope · Ant–aphid interaction · Context dependency · Nutrient transfer · Trophic level

Introduction

Interspecific interaction, a key component of biodiversity and ecosystem function, is a central issue in ecology (Palmer et al. 2008), and ant–aphid mutualism is a model system for the study of the ecology and evolution of this interaction (Heil and McKey 2003). Although ants are small, because they are predators with a wide distribution and include a large variety of species, they play very important roles in terrestrial ecosystems (Holldobler and Wilson 1990). Aphids excrete honeydew, an important food resource for ants (Buckley 1987; Rico Gray 1993). Honeydew has a high concentration of carbon, and extremely low concentration of nitrogen, which leads to an increased demand for nitrogen to balance the carbon–nitrogen ratio. Therefore, ants serve as predators of aphids’ natural enemies and predators of other herbivores on leaves as well (Stadler and Dixon 2005). Ant-attended aphids excrete honeydew that has a higher concentration of amino acids, sucrose, and trehalose than do those of unattended aphids (Yao and Akimoto 2001). In addition, aphids can change the quality of their honeydew, which affects their mutualism with ants (Johansson and Gibb 2012). Studies have reported the effects of ant–aphid mutualism on plants, in which the absence of ants reduced the quantity of plant seeds and increased herbivore and leaf loss (Rosumek et al. 2009; Sipura 2002; Trager et al. 2010; Wimp and Whitham 2001).

In direct ant–plant mutualism, in which plants provide food or nesting places for ants, and in return, ants protect the plants from herbivores and supply nutrients to their host

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plants. Ants accumulate organic matter in their nests (e.g., leaf pouches, domatium, spongy-looking galleries, and petiolar chambers) by storing food, discarding debris, and defecating, and these activities may provide nutrients to the host plants (Defosse et al. 2009; Solano and Dejean 2004; Treseder et al. 1995). Using stable isotope analysis, Fischer et al. (2002) found up to 25% nitrogen transfer from ants to plants within 6 days, and Treseder et al. (1995) calculated that 39% of carbon and 29% of nitrogen in host plant leaves are derived from ant debris in the leaf cavities. Wagner and Nicklen (2010) tested the ant-derived nutrients assimilated by *Acacia constricta*, and found that 24 days after feeding ^{15}N -labeled food to *Dorymyrmex* sp. (Smithi complex) workers nesting near *A. constricta*, the ^{15}N -labeled plant leaves contained significantly higher levels of ^{15}N than did those of *A. constricta* without an associated nest. It remains unknown whether a similar mechanism exists in indirect ant–plant mutualism (e.g., an ant–aphid–plant system). Soil-dwelling ants also deposit debris or defecate in and near their nests, and as a result, the soil near the nests becomes enriched with organic matter (Wagner and Nicklen 2010). Further, studies have reported higher nutrient cycling rates in soil associated with ant nests compared to those without (Jurgensen et al. 2008). *Lasius fuliginosus* usually nest in large plant roots or trunks where they may provide nutrients to host plants (Kalnihs and Dreimanis 2007). Therefore, we wished to test whether a nutrient transfer mechanism also exists in indirect *L. fuliginosus*–*Q. liaotungensis* mutualism.

Because stable isotopes are not hazardous, they are a simple, practical, and highly accurate method to use in these studies (Yi and Zhang 2005). Because $\delta^{15}\text{N}$ provides constant enrichment (approximately 3.4‰) as the trophic level increases, ^{15}N stable isotopes are powerful tools with which to evaluate the structure and dynamics of ecological communities (Post 2002). Analyzing ants' trophic level can reflect relationships among the organisms in the food web indirectly. For example, the trophic level of *Solenopsis invicta* is higher in their native country of Argentina, which implies that *S. invicta* forage there primarily as carnivores. However, when *S. invicta* invaded the United States, they established mutualism with honeydew-producing insects and occupied a lower trophic level (Gibb and Cunningham 2011). Because honeydew is a plant-based food source, the more the ants depend on honeydew, the lower their trophic level (Davidson et al. 2003). This change in the ants' trophic level also may affect nutrient transfer in the food web (Wilder et al. 2011).

In our previous research, we found that the altitudinal distribution of ant and aphid abundance differed significantly in the Dongling Mountain (the study area in this research), where the abundance of *L. fuliginosus* and the aphid, *Lachnus tropicalis*, at low altitudes was significantly

higher than that at high altitudes (Zhang et al. 2015). These results provided the conditions necessary to study the context dependency of ant–aphid mutualism. Therefore, to understand this dependency, we used the ^{15}N stable isotope method in this work to explore the ecological processes in the ant–aphid–plant system with respect to the ants' trophic level and nutrient transfer to determine whether the effect of nutrient transfer and the ants' trophic level varied with altitude.

Materials and methods

Study area

The study took place at the Beijing Forest Ecosystem Research Station located on Dongling Mountain (30°057'29N, 115°025'33E), which is part of the Chinese Ecological Research Network (CERN). This area has a warm, temperate, continental monsoon climate with a mean annual precipitation of 500–650 mm, and an average annual temperature of 5–10 °C (Zhang et al. 2012). The annual frost-free period is approximately 195 days, with approximately 2600 h of sunshine. The altitude in most of the area is greater than 1000 m above sea level, with the highest peak at 2303 m. The study was conducted in an 80-year-old oak (*Q. liaotungensis*)-dominated secondary forest. *Q. liaotungensis* grows primarily from 1020 to 1770 m on sunny, semi-sunny, and semi-shady slopes, and usually is mixed with *Betula platyphylla* and *Populus davidiana* (Zhang et al. 2006).

Experimental design

The study was conducted at two sites: one at low altitude (1270–1360 m) and one at high altitude (1460–1560 m). At each site, three ant nests were designated as the control group and three were assigned to the treatment group. A ^{15}N -feeding experiment was performed in July and September 2014. Ants in the treatment group were fed ^{15}N -labeled glycine (98 at.% ^{15}N) aqueous honey solution (30% w/v, three times) for 15 days. We buried tinfoil bowls at ground level or slightly below, each of which contained 200 ml of aqueous honey solution (totaling 5 g ^{15}N -glycine for each nest). During labeling, the bowls were protected from rainfall with an inverted bowl and fixed with metal wires. Before and after the last were labeled at 5, 10, 20, 30, and 60 days, ants, soil, and leaves were sampled, respectively. Approximately 80–100 individual ants were collected from the trees. We cut three *Q. liaotungensis* twigs and picked six ripe leaves from each. The results showed that the ^{15}N stable isotope enriched the soil primarily within 0–30 cm and accounted for approximately 70–85% within 0–100 cm

(Yu et al. 2012). Therefore, five samples of soil surface grass and leaf litter were collected at each site within a radius of 1 m of the nest, after which approximately 10 g of soil taken within 15–20 cm was put into Ziploc bags. These samples were dried at 60 °C for 48 h and kept dry until further analysis.

Isotopic analysis

Dried leaves and soil were ground into fine powder with a ball mill; the ants' abdomens were removed to avoid the influence of gut contents on the ^{15}N values (Tillberg et al. 2006), and then were ground to powder with an agate mortar and pestle. Three leaf and five soil samples were mixed to ensure that the samples were homogeneous and representative. Samples weighing 1–20 mg were packed into tin capsules for isotopic analysis, and a total of 216 samples were analyzed. The ratio of heavy-to-light isotopes of N for all samples was measured using a continuous-flow isotope-ratio mass spectrometer (Finnigan Delta V Advantage, Finnigan MAT, San Jose, CA, USA) at the Stable Isotope Laboratory. The nitrogen stable isotope composition was expressed as δ (‰): $\delta^{15}\text{N}$ (‰) = $[(R_{\text{sample}}/R_{\text{standard}}) - 1] \times 1000$, where R is the ratio of $^{15}\text{N}/^{14}\text{N}$ of the sample and standard, and the isotopic standard for nitrogen is atmospheric N_2 (Fischer et al. 2002; Gibb and Cunningham 2011).

Analysis of ants' trophic level

We used stable isotope analysis to estimate the ants' trophic level. In terrestrial ecosystems, roots, leaf litter, and herbivores are used commonly as a baseline (Ponsard and Arditi 2000). In our study, $\delta^{15}\text{N}_{\text{ant}}$ and $\delta^{15}\text{N}_{\text{leaf}}$ values from control nests were used to estimate ants' trophic level. The simplest model for estimating the trophic level of a secondary consumer is $\text{trophic level} = \lambda + (\delta^{15}\text{N}_{\text{secondary consumer}} - \delta^{15}\text{N}_{\text{base}}) / \Delta\delta^{15}\text{N}$, where λ is the trophic level of the organism used to estimate $\delta^{15}\text{N}_{\text{base}}$ (e.g., $\lambda = 1$ for primary producers); $\delta^{15}\text{N}_{\text{secondary consumer}}$ is measured directly; and $\Delta\delta^{15}\text{N}$ is the fractional isotope values of adjacent trophic levels. All consumer levels were assumed to span 3.4‰. In our study, the ants' trophic level was calculated as follows: $\text{trophic level} = (\delta^{15}\text{N}_{\text{ant}} - \delta^{15}\text{N}_{\text{leaf}}) / 3.4\text{‰} + 1$ (Post 2002).

Field survey

We surveyed the abundance of terrestrial and arboreal ants, aphids, and the DBH of trees in all sites, and used pitfall traps to investigate the abundance of terrestrial ants, as this is a sampling method used extensively for surface-foraging invertebrates (Cardoso et al. 2007; Wardle et al. 2011). Small 20 m $\times$ 20 m plots were established at 2.5 m

from the plot boundary, and a beaker (diameter = 7.9 cm, depth = 9.7 cm) was buried as a trap every 5 m at ground level or slightly below. Each beaker contained 50 ml of 5% alcohol, and once every month from May to September 2009, we collected the ants after 48 h and recorded their total number. In order to reduce the boundary effect, we established large plots of 80 m $\times$ 60 m in 2010 and 2011, and buried beakers only in the middle of the plots. The abundance of terrestrial ants and aphids, and the DBH of trees were measured in August 2013. For each nest, ten trees were selected that included three feeding routes in nine trees and the nest tree. Based on the nearest, farthest, and intermediate distances of each feeding route to the nest tree, we recorded a cross section of ants on every tree within 1 min. To examine aphid abundance, we cut off three twigs randomly and recorded the number of aphid on 18 leaves. We measured the DBH of trees 1.3 m from the ground with a diameter tape. During the experiment, soil temperature was recorded automatically every hour by means of a button thermometer buried 10 cm below the surface.

Statistical analysis

We used *t* tests to determine the differences between the control and treatment ants, soil, and leaves, and the data were ln-transformed to reduce heterogeneity of variance; the ants' trophic level, tree DBH, and soil temperature at different altitudes were analyzed similarly. SPSS 19.0 (SPSS, Chicago, IL, USA) was used for all analyses, and differences were considered significant at $p < 0.05$. Related graphics were completed in Origin9.0.

Results

For the ^{15}N -labeled experiment, Table 1 shows the differences between treatments and controls in the $\delta^{15}\text{N}$ of ants, soil, and leaves at high- and low-altitude sites. As seen in the table, before labeling (0 day), there were no significant differences in the $\delta^{15}\text{N}$ values of ants and soil between the groups ($p > 0.05$), while after labeling (20, 25, 35, 45, and 75 days), there were significant differences ($p < 0.05$). The $\delta^{15}\text{N}$ of values ants and soil in the treatment groups were significantly higher than those in the controls, both at the high- and low-altitude sites (Fig. 1a, b). The $\delta^{15}\text{N}$ of leaves yielded similar results at low-altitude sites, but there was a significant difference in $\delta^{15}\text{N}$ in leaves between treatments and controls at high-altitude sites before labeling. To eliminate this significant difference, we analyzed the relative increment of $\delta^{15}\text{N}$ in leaves ($\delta^{15}\text{N}$ of control and treatment leaves after labeling minus $\delta^{15}\text{N}$ of leaves before labeling, respectively). Table 2 shows that after labeling,

Table 1 Differences of ants', soil's, leaves' $\delta^{15}\text{N}$ values between treatments and controls in high-altitude and low-altitude sites

Variables	Time (days)	df	T_1	P_1	T_h	P_h
Ants	0	1, 4	1.484	0.212	-0.423	0.694
	20	1, 4	-16.629	0.000	-14.125	0.000
	25	1, 4	-21.606	0.015	-22.403	0.000
	35	1, 4	-12.806	0.000	-7.734	0.002
	45	1, 4	-4.036	0.016	-14.547	0.000
	75	1, 4	-5.902	0.004	-7.199	0.002
Soil	0	1, 4	0.616	0.571	-2.090	0.105
	20	1, 4	-4.915	0.008	-11.579	0.000
	25	1, 4	-4.665	0.010	-5.490	0.005
	35	1, 4	-2.783	0.050	-4.205	0.014
	45	1, 4	-2.770	0.050	-5.745	0.005
	75	1, 4	-5.614	0.005	-5.742	0.005
Leaves	0	1, 10	-1.335	0.211	-3.328	0.008
	20	1, 10	-2.324	0.042	-6.434	0.000
	25	1, 10	-3.083	0.012	-7.535	0.000
	35	1, 10	-2.679	0.029	-5.609	0.000
	45	1, 10	-2.909	0.016	-5.614	0.000
	75	1, 10	-2.742	0.021	-7.788	0.000

P_1 : significance between treatments and controls in low-altitude sites, P_h : significance between treatments and controls in high-altitude sites

Fig. 1 Differences of variables along the altitude. **a** Difference of ants' $\delta^{15}\text{N}$ between treatments and controls in high-altitude and low-altitude sites; **b** difference of soil's $\delta^{15}\text{N}$ between treatments and con-

trols in high-altitude and low-altitude sites; **c** difference of relative incremental of leaves' $\delta^{15}\text{N}$ between treatments and controls in high-altitude site. Error bars represent SEs

the difference in $\delta^{15}\text{N}$ of leaves between controls and treatments was significant at high-altitude sites ($p < 0.05$), in that the relative increment in $\delta^{15}\text{N}$ of leaves at treatment sites was significantly higher than that of controls (Fig. 1c).

To explore whether the effect of nitrogen transfer differed with altitude, we analyzed the relative increment of

$\delta^{15}\text{N}$ in ants, soil, and leaves. Table 3 shows that there was no significant difference in the relative increments of $\delta^{15}\text{N}$ in ants and leaves between the treatment and control groups at high- and low-altitude sites ($p > 0.05$), but the difference in soil's $\delta^{15}\text{N}$ was significant ($p < 0.05$). As shown, the relative increment of $\delta^{15}\text{N}$ in soil in

Table 2 Differences of the relative incremental leaves' $\delta^{15}\text{N}$ between treatments and controls in high-altitude sites

Leaves _{relative incremental} (days)	<i>df</i>	<i>T</i>	<i>P</i>
20	1, 10	−4.094	0.002
25	1, 10	−5.152	0.000
35	1, 10	−3.440	0.006
45	1, 10	−3.411	0.007
75	1, 10	−2.908	0.016

Table 3 Differences of the relative increments of ants', soil's, leaves' $\delta^{15}\text{N}$ between treatments in high-altitude and low-altitude sites

Variable	<i>df</i>	<i>T</i>	<i>P</i>
Soil	1, 28	−4.158	0.000
Ants	1, 28	−0.581	0.566
Leaves	1, 58	−0.543	0.589

high-altitude treatments was significantly higher than that at low altitudes.

We also analyzed the altitudinal variation in the trophic level of ants from control nests between high and low altitudes, and found that their trophic level at high altitude was significantly lower than that at low altitude ($t_{1,40}=2.55$, $p<0.05$). The DBH of trees at high altitude also was significantly larger than that at low altitude ($t_{1,118}=-2.09$, $p<0.05$). However, although it was slightly lower at high-altitude (14.01 ± 0.39) than that at low-altitude sites (15.08 ± 0.47), the difference in soil temperature was not significant ($t_{1,52}=1.77$, $p>0.05$).

Discussion

We performed a ^{15}N -labeled experiment to examine the effects of nitrogen transfer in indirect ant–plant mutualism. Results showed that after labeling, the $\delta^{15}\text{N}$ of ants, soil, and leaves were significantly higher than those before labeling. This demonstrated that the debris and feces that ants deposit in belowground nests enrich the soil organic matter—the result that is consistent with previous findings (Wagner and Nicklen 2010). According to these results, nutrient transfer from ants to plants may occur both through soil and by ants' supplying nutrients directly. Because the belowground system is complex, its role in the transfer process of animals in the soil, microorganisms, and roots remains unclear. Therefore, future studies should focus on the process of nutrient transfer and quantify the value of the contributions from ants to plants.

At our treatment sites, the $\delta^{15}\text{N}$ of soil differed significantly at high and low altitudes, which suggests that the effect of nutrient transfer on ant–aphid mutualism varies

with altitude. Studies have reported that soil temperature may influence the isotope fractionation of mineralization, nitrification, and denitrification in nitrogen cycling and affect the activity of soil microorganisms. For example, when the temperature increased, the activities of the soil nitrobacteria and ammonifiers were strengthened, such that light isotopes reacted faster, and thus enriched more heavy isotopes (Zhong 2013). Nevertheless, in this study, we found that soil temperature was slightly higher at low altitudes, and soil's $\delta^{15}\text{N}$ at high altitudes was significantly higher, which suggests that soil temperature may not affect the nitrogen transfer in ant–plant mutualism significantly. In addition, temperature and precipitation are important environmental factors that affect leaves' $\delta^{15}\text{N}$ (Martinelli et al. 1999; Zhou et al. 2012). Previous studies in the Dongling Mountain have reported a significant negative relationship between tree leaves' $\delta^{15}\text{N}$ and precipitation, but a significant positive relationship with annual temperature (Liu et al. 2009). Thus, nutrient transfer should be explored with respect to both biotic and abiotic factors.

Plant size is an important factor that affects the ant–plant relationship. *L. fuliginosus* prefer to nest in larger trees, which therefore may play an important role in mutualism (Zhang et al. 2012). Results have shown that trees' DBH is significantly greater at high altitude, but the $\delta^{15}\text{N}$ of leaves is not. This indicates that larger plants may not promote nitrogen transfer in indirect ant–plant interactions. This is consistent with the findings that tree size does not influence the ecological effects of indirect ant–plant interactions (Zhang et al. 2012).

The more the ants depend on plant-based foods (e.g., honeydew), the lower their trophic level. Our work showed that ants' trophic level at high altitudes was significantly lower than that at low altitudes. This indicates that ants at higher-altitude sites depend more on plant-based food (e.g., honeydew), which suggests that there may be a greater degree of ant–aphid mutualism at high altitudes. Recent studies have shown that higher ant abundance is related to lower trophic level as well (Davidson et al. 2003; Zhang et al. 2015). In contrast to previous studies, our results showed that a higher trophic level was associated with higher ant abundance at low altitudes. This could happen if there is a shortage of protein-based food (e.g., herbivores), in which case ants may eat their mutualist partner, the aphid, to maintain a balanced nitrogen–carbon diet (Petty et al. 2012). At the lower-altitude site, the higher abundance of ants demands more protein-based foods. When there is a shortage of other sources of protein-based foods (other herbivores than aphids), ants may eat aphids to balance the nitrogen–carbon ratio of their bodies. This will decrease the aphids' population and the honeydew production as well. As a result, the food sources of ants at low-altitude sites would have more nitrogen-based than plant-based food

(honeydew). Unfortunately, we did not consider the aphid-eating phenomenon in this work, but believe it deserves further investigation.

The results of our ^{15}N -labeled experiments at different altitudes varied, which suggests that ant–aphid mutualism might be context dependent and affect nutrient transfer in the food web. Thus, our proposed context dependency of ant–aphid mutualism may be an important factor that influences altitudinal variations in nutrient transfer. In this study, we did not address the functions in nutrient transfer of roots and animals in the soil. The transfer process in the belowground system is complex, and future studies must pay careful attention to the specific transfer process and consider the effects of abiotic factors on nutrient transfer.

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