

Article

Effects of Large-Scale Nitrogen Fertilization on Insect–Plant Interactions in the Canopy of Tall Alder Trees with N₂-Fixing Traits in a Cool Temperate Forest

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Abstract: Nitrogen (N) deposition is expected to influence forests. The effects of large-scale N fertilization on canopy layer insect–plant interactions in stands of tall, atmospheric nitrogen (N₂)-fixing tree species have never been assessed. We conducted a large-scale fertilization experiment (100 kg N ha⁻¹ year⁻¹ applied to approximately 9 ha) over three years (2012–2014) in a cool temperate forest in northern Japan. Our goal was to evaluate relational responses between alder (*Alnus hirsuta* [Turcz.]) and their insect herbivores to N deposition. Specifically, we assessed leaf traits (N concentration, C:N ratio, condensed tannin concentration, and leaf mass per unit area (LMA)) and herbivory by three feeding guilds (leaf damage by chewers and the densities of gallers and miners) between the fertilized site and an unfertilized control. Fertilization led to increased galler density in spring 2013 and increased leaf damage by chewers in late summer 2014. For leaf traits, the LMA decreased in spring 2013 and late summer 2014, and the C:N ratio decreased in late summer 2013. The N and condensed tannin concentrations remained unchanged throughout the study period. There was a negative correlation between LMA and leaf damage by chewers, but LMA was not correlated with galler density. These results show that large-scale N fertilization had a positive plant-mediated (i.e., indirect) effect on leaf damage by chewers via a decrease in LMA in the canopy layer. Changes in physical defenses in canopy leaves may be a mechanism by which N fertilization affects the herbivory in tall N₂-fixing trees.

Keywords: *Alnus hirsuta*; forest communities; insect herbivory; nitrogen deposition; leaf traits; physical defenses



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1. Introduction

The effects of enhanced atmospheric nitrogen (N₂) deposition, a result of increased anthropogenic activities, on terrestrial ecosystem processes is a growing environmental problem [1,2]. Enhanced deposition leads to changes in plant community composition, decreases in plant diversity, and altered nitrogen (N) cycling [3–6]. There are several potential mechanisms by which deposition affects terrestrial ecosystem processes. Deposited N can act as a fertilizer, directly stimulating plant growth and increasing the N content in plant tissue; many studies have focused on this phenomenon [7]. On the other hand, N deposition may indirectly affect plant communities and terrestrial ecosystems by modifying plant–insect interactions [7,8]. Because increased N deposition can affect ecosystem processes directly (e.g., nutrient cycling [9]) or indirectly (e.g., causing shifts in herbivory resulting from altered host plant traits), more consideration should be given to changes in plant–insect interactions following increased N deposition, in order to fully understand N deposition effects on terrestrial ecosystem processes.

Many studies have shown that shifts in leaf traits, such as an increase in foliar N content and/or a decrease in carbon (C)-based chemical defenses, which can be caused by increased N deposition, positively affect the performance and/or abundance of insect herbivores, including Lepidoptera, Hymenoptera, and Coleoptera [10–14]. However, these studies have been based on small-scale ecosystem manipulations [10,12,15]. Experiments employing open-top chambers and N fertilization in <10 m × 10 m field plots [10,12,15] have important limitations, such as the lamp effect, wherein insects that prefer the conditions of a treated site (e.g., moths) congregate at an artificially high density, while others avoid the site due to those same conditions [16]. Thus, extrapolating data obtained from small-scale experiments to predicting the effects of these changes at large scales will not yield robust results. To avoid these limitations, N deposition studies should focus on large-scale manipulations and examine how N fertilization affects the performance and/or abundance of insect herbivores [17,18].

Plant tissues often have much lower N levels than herbivorous insect tissues [19]. N-containing nutrients in plant tissues are often a limiting factor for insect herbivores, either because of limited resource utilization, a high population density, or both [13,20]. According to the C-nutrient balance (CNB) hypothesis, when N is abundant, more C is allocated to growth than to C-based chemical defenses (e.g., condensed tannins and total phenolics) in plants [21,22]. Thus, the majority of N deposition studies have focused solely on changes in nutrients and C-based chemical defenses. However, N fertilization could also affect the hardness and thickness of foliage (i.e., leaf mass per unit area (LMA)) because elevated N enhances photosynthesis more than growth; thus, LMA may decrease [23]. It is probable that N fertilization could lead to shifts in leaf traits to the advantage of insect herbivores, not only by decreasing chemical defenses, but also by reducing physical defenses—specifically, the toughness and thickness of leaves. LMA has been shown to be a significant component of leaf toughness. Leaf toughness is widely recognized as one of the most effective forms of physical defense in leaves [24,25].

Numerous N fertilization experiments were conducted using N-limited species, which indicated that shifts in foliar chemical and nutritional status could affect plant–insect interactions, specifically with insect herbivores [7]. Alder (*Alnus*) species have a greater potential to accumulate N (mg/g) in their tissues than other species, owing to their N₂-fixing traits, in association with symbiotic *Frankia* species (alder, >25 mg/g; oak, 15–25 mg/g; pine, 12–17 mg/g) [26]. Alder species are typically the largest natural pathway for N input in certain forest types [27,28]. Given the research focus on N-limited species, it remains unclear how the leaf traits of N₂-fixing plants may be affected by increased N deposition, and how, in turn, plant–insect interactions may be affected, even when N fertilization promotes plant growth [29,30].

Classifying herbivorous insects into ecologically and evolutionarily related feeding guilds [31] allows for comparisons and generalizations that cannot be made using taxonomic groups alone [9,32]. In an insect herbivore community, chewers are external herbivores and gallers and miners are internal herbivores. These guilds (i.e., chewers, gallers, and miners) may respond differently to N fertilization. For example, N fertilization was shown to affect chewer performance (body size, development time, and survival) as a result of altered plant traits (increased N content and decreased C-based chemical defenses) [7]. By contrast, other studies showed no effect of N fertilization on the density and growth rate of the galler *Eurosta solidaginis* [33], and the abundance and diversity of miners [34]. A thorough understanding of insect herbivore responses to N fertilization requires assessing the responses of major feeding guilds on the same host plant.

Here, we report the first manipulative test of large-scale N fertilization effects on alder, an N₂-fixing species, and its insect herbivores. We aimed to determine how fertilization affects leaf traits in canopy-sized trees and their interactions with insect herbivores. We asked how large-scale fertilization influences leaf traits in mature alder, how fertilization affects the three major insect feeding guilds, and if certain interactions between leaf traits and feed guilds are affected by N fertilization.

2. Materials and Methods

2.1. Site Description and Study Species

This study was conducted in Tomakomai Experimental Forest, Hokkaido University, Japan (42°40' N, 141°36' E). The forest is formed on a 2 m deep volcanic ash layer that accumulated following the eruptions of Mt. Tarumae in 1699 and 1739; soils are very shallow [35]. The study area was divided into control (~10 ha) and treatment (fertilized) (~9 ha) compartments (sites). Tree basal area was investigated in four plots (20 m × 20 m) at each site; we found no difference between the control ($27.5 \pm 4.6 \text{ cm}^2/\text{m}^2$) and the fertilized sites ($24.8 \pm 4.6 \text{ cm}^2/\text{m}^2$) using a *t*-test ($t = 0.827$; $p = 0.440$). The dominant tree species at the study site were *Quercus crispula*, *Acer mono*, *Sorbus alnifolia*, and *Tilia japonica* [36]. Mature tall alder (*Alnus hirsuta* [Turcz.]) trees were the focus of our study. The alder trees were not suppressed by surrounding trees, and the distances between the trees were more than 10 m. The buds typically open in early spring (late April or early May) and the leaves emerge twice per year—in spring (early leaves) and in late summer (late leaves) [37].

2.2. Large-Scale N Fertilization Experiment

The fertilized site (~9 ha) was fertilized with a urea solution ($100 \text{ kg N ha}^{-1} \text{ year}^{-1}$; $\text{CO}[\text{NH}_2]_2$) designed for dry application; it was applied to simulate a pulse of N input as rain melts the urea into the soil. The fertilizer was distributed by helicopter in 2013 (April 15 and 16) and manually in 2014 (April 21 and 22). Akata et al. [38] found that the N fertilization experiment had a larger diameter and high nitrogen concentrations in fine roots of oak species and dissolved organic carbon in soil. We did not have site replications, because individual trees could be considered as independent within and between the treatments. When conducting field experiments, the best way to do this is to create replications of sites, but if this is difficult to conduct such as in large-scale experiments, and individuals can be considered as replications, this can be another option [39,40]. Moreover, we had a limited number of alder trees in the study site, although it was worth-studying them due to their specific N_2 -fixing traits.

2.3. Estimation of Leaf Traits

We assessed 10 mature (>10 m in height) alder trees (diameter at breast height (DBH) = $30.7 \pm 7.5 \text{ cm}$ in the control site ($n = 5$) and $24.0 \pm 4.6 \text{ cm}$ in the fertilized site ($n = 5$) growing along roads through the control and fertilized compartments. One tree from the fertilized site died in 2014 and was thus excluded. During each sampling period, two branches (1 cm in diameter) were randomly sampled from the canopy of each tree using a crane car. All branches were located on the top of the canopy and were fully exposed to sunlight. Samples were collected from the selected trees from 2012–2014, including a visit on June 28–29, 2012, prior to N fertilization, which was used as a preliminary assessment period. Otherwise, sampling was conducted during early and late periods in each year, on June 17–18 and September 17–18, 2013, and June 19–20 and August 27–28, 2014, respectively, because the seasonal fluctuation of moth communities including chewers and galls showed two large peaks in mid-June and early to mid-August in our study site [41].

Leaf traits (N concentration, C:N ratio, condensed tannin concentration (a measure of C-based defense), and LMA) were determined from at least five leaves randomly collected from the two sampled branches from each tree. LMA was calculated using:

$$\text{LMA} = W_{dry} / A_{area}$$

where W_{dry} is the mass of leaves dried at 60 °C for 48 h, and A_{area} is the leaf area, calculated from leaf disks obtained by leaf punching (1.5 cm in diameter). Dried samples were maintained in an oven at 40 °C for another week, then ground to a fine powder using an analytical mill with a micro-smasher to assess leaf traits. C:N ratio was used as an indicator of C-based chemical defenses [21]. The leaf N concentration and C:N ratio were determined using an NC analyzer (Sumigraph NC-900; Sumika Chemical Analysis Service, Osaka,

Japan). The condensed tannin concentration was determined using the method described by Julkunen-Tiitto [42].

2.4. Insect Herbivore Feeding Guilds

We collected 364 individuals accounting for 33 species of Coleoptera, Hemiptera, Hymenoptera, and Lepidoptera (Table S1).

Three major feeding guilds (chewers, gallers, and miners) were used to assess the community-level response to N fertilization. Feeding guilds are closely related to host specificity: chewers are generalist herbivores, whereas gallers and miners are specialists [43]. The three feeding guilds were assessed in 10 randomly selected current-year shoots obtained from the two canopy branches (diameter 1 cm) taken from each tree. First, leaf damage by chewers was defined as the percentage of leaf area chewed with respect to the total leaf area and was scored visually using six categories: 0% = 0, 1–10% = 1, 11–25% = 2, 26–50% = 3, 51–75% = 4, and 76–100% = 5 [44,45]. The median of each group was used in the statistical analysis of leaf damage by chewers (i.e., 0, 5, 18, 38, 63, and 88%, respectively). We then counted the number of gallers and miners on each shoot to determine their respective densities.

2.5. Statistical Analyses

Differences in leaf traits (N concentration, C:N ratio, condensed tannin concentration, and LMA) and guild-level insect herbivory (leaf damage by chewers and the respective densities of gallers and miners) were compared between the control and fertilized sites before and after N fertilization using *t*-tests. Pearson's correlation coefficient was used to assess correlations between leaf traits and feeding guilds that were significantly altered by N fertilization. All variables were tested for normality using the Shapiro–Wilk test, and non-normal variables were log-transformed. All statistical analyses were conducted using R version 2.9.2 [46].

3. Results

3.1. Effect of N Fertilization on Leaf Traits

Prior to the first application of N in early 2012, there were no significant differences in any of the leaf traits between the control and fertilized treatment ($p > 0.050$, *t*-test; Figure 1). Following N fertilization, there was a significant decrease in C:N ratio in late 2013 ($t = 3.537$, $p = 0.007$, Figure 1B), and in LMA in early 2013 ($t = 2.610$, $p = 0.003$, Figure 1C) and marginally in late 2014 ($t = 1.982$, $p = 0.080$, Figure 1C). There were no significant differences in N concentration ($p > 0.050$, Figure 1A) and condensed tannin concentration ($p > 0.050$, Figure 1D) between the control and fertilization treatment throughout the study period, which included two years following N fertilization.

3.2. Insect Herbivore Responses

As with leaf traits, no significant differences in leaf damage and galler or miner density were detected between the control and fertilization site prior to N fertilization ($p > 0.050$, Figure 2). Following N fertilization, there was a significant increase in leaf damage by chewers in late 2014 ($t = -2.571$, $p = 0.037$, Figure 2A). Galler density decreased marginally in early 2013 ($t = 2.221$, $p = 0.057$, Figure 2B). There was no difference in miner density between the control and fertilized sites throughout the study period ($p > 0.050$, *t*-test; Figure 2C).

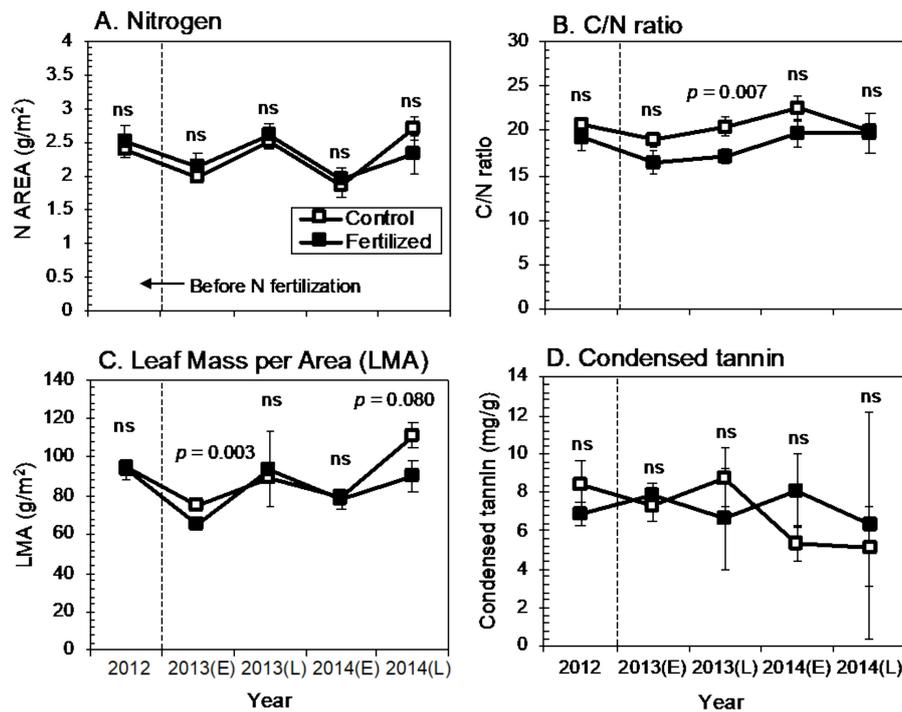


Figure 1. Changes in leaf nutrients of alder trees. (A) Nitrogen content, (B) C/N ratio, (C) leaf mass per area (LMA), and (D) condensed tannin in the leaves of alder trees at the control (empty boxes, $n = 5$) and fertilized (filled boxes, $n = 5$) sites from 2012 (before N fertilization) to 2014. E and L indicate early and late seasons. Each point is mean \pm SE and the p -values are the results of t -tests (ns, $p > 0.05$).

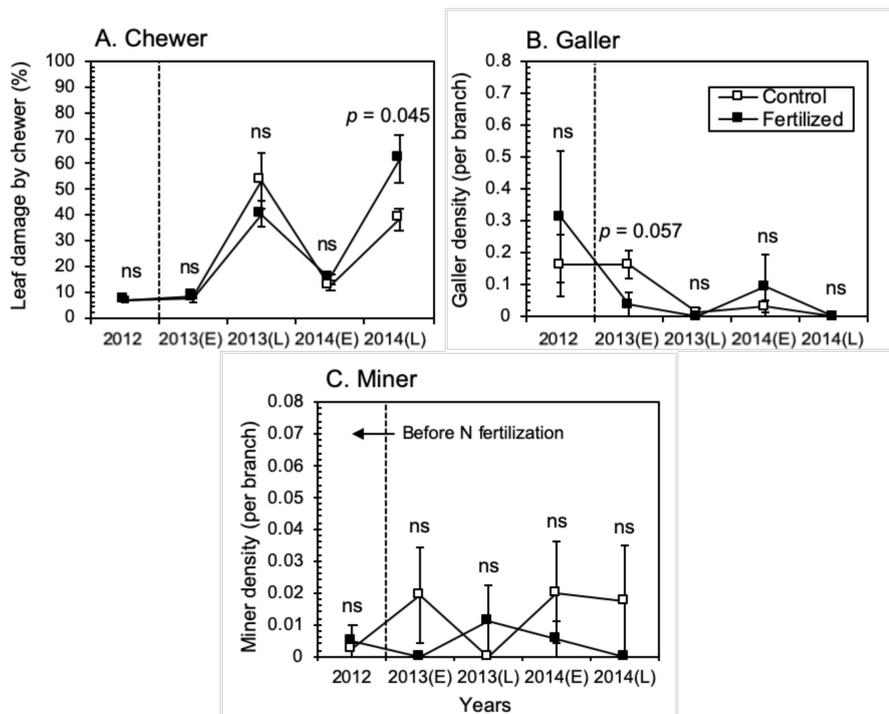


Figure 2. Changes in the three major guilds of insect herbivores categorized based on feeding type. (A) Leaf damage by chewers, (B) galler density, and (C) miner density on trees in control (empty boxes, $n = 5$) and fertilized (filled boxes, $n = 5$) sites from 2012 (before N fertilization) to 2014. E and L indicate early and late seasons. Each point is mean \pm SE and the p -values are the results of t -tests (ns, $p > 0.05$).

3.3. Correlation Analysis between Leaf Traits and Insect Herbivory

We assessed correlations in the variables (LMA in early 2013 and late 2014, C:N ratio in late 2013, leaf damage by chewers in late 2014, and galler density in early 2013) that were significantly or marginally altered following N fertilization. There was a negative correlation between LMA and leaf damage by chewers in late 2014 ($r = -0.887$, $p = 0.001$, Figure 3). However, LMA was not correlated with galler density in early 2013 ($p > 0.05$). The timing of changes in C:N ratio and herbivory by any feeding guild did not align in any sampling period.

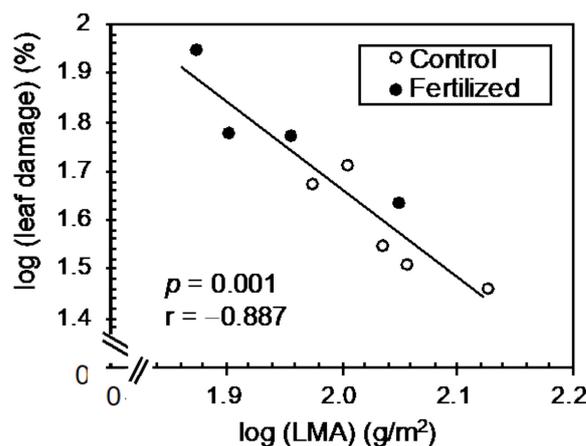


Figure 3. Pearson's correlation between the LMA and leaf damage by chewers in late 2014. Circles in white and black indicate control and fertilized trees, respectively. Regression results and the line of best fit are indicated.

4. Discussion

Large-scale N fertilization led to a decrease in the C:N ratio and LMA in mature *A. hirsuta*, an N₂-fixing species, and an increase in leaf damage by chewers and a decrease in the density of gallers, at least once during the study period; although other leaf traits (i.e., N and condensed tannin concentrations) remained unchanged throughout the study period. We found a negative relationship between LMA and leaf damage by chewers, but LMA was not correlated with the density of gallers. Our results suggest that large-scale N fertilization may have a positive, indirect (plant-mediated) effect on leaf damage by chewers, via a decrease in physical defense (LMA) in the canopy leaves of mature alder trees.

Interestingly, although we found no effect on N and condensed tannin concentrations, Koike et al. [15] reported that N addition led to a decrease in C:N ratio and chemical defense compounds, such as total phenolics and condensed tannin, in alder saplings. This discrepancy may be related to maturity and plant size. The responses of woody plants to shifting site conditions may vary depending on their stage of development [47–50]. In *Q. crispula*, N fertilization was shown to influence leaf traits (e.g., N, C:N, and condensed tannins) in two-year-old seedlings [15], but not in mature trees [51]. Given that our study employed a large-scale manipulation, which avoided issues such as the lamp effect [16], our findings suggest the actual effects of large-scale global change in N deposition [52]. However, no clear conclusions on the responses of secondary defense compounds can be drawn by the present study, because only tannins were measured, and future research should include a broad range of secondary metabolites.

LMA has been shown to decrease following N fertilization [13,52], especially when plant nutrients are limited [53,54]. In our study, N fertilization decreased the LMA of *A. hirsuta* (N₂-fixing species) (Figure 1). Ruess et al. [55] also reported that N-fertilization significantly decreased leaf thickness of thin-leaf alder (*A. tenuifolia*), due to the down-regulation of N-fixation. The decrease in LMA may be driven primarily by a shift from

N to C limitation in the fertilized site—carbon allocated in leaf growth is partitioned to increase leaf area though the production of thinner leaves.

Lee et al. [56] showed that large-scale N fertilization enhanced leaf quality (e.g., increase in N concentration and decrease in C:N ratio) in mature oak trees >10 m in height. These results are contrary to our findings with *A. hirsuta* (only C:N ratio), and may be reflective of differences in the severity of N deficiency between N-limited and N₂-fixing species. N-limited species may be more sensitive to N fertilization than N-fixing species because of more N deficiency. Furthermore, leaf density and/or thickness reflect variation in anatomical structure arising from environmental conditions and species traits [57], although the role of physiological regulation in determining leaf traits remains unclear [23].

Major feeding guilds of insect herbivores may respond differently to N deposition-induced changes in the quality of their host plants [20]. Most previous studies have suggested that N fertilization affects chewer insects by altering chemical and nutritional qualities [10–14], whereas gallers and miners have been more poorly studied [13]. To assess insect herbivore responses to N fertilization, we assessed how each of the three major feeding guilds were affected on the same host plants and found that large-scale N fertilization significantly increased leaf damage by chewers (Figure 2). Leaf damage was negatively associated with LMA (Figure 3). As a physical defense, LMA has been reported to influence the performance of chewers [13,54,58,59]. These results suggest a mechanism by which large-scale N fertilization affects leaf damage by chewers on mature alder—through changes in physical defenses. Additionally, our results indicate that N fertilization marginally decreased galler density (Figure 2), as opposed to Hartley and Lawton's findings [33], which suggested that leaf N concentration was not correlated with the density of the gall midge *Eurosta solidaginis*. It appears that N deposition-related changes in plant quality did not affect galler density because density was not associated with any of the assessed leaf traits. Several review papers have suggested the possibility of N deposition-mediated changes in herbivore susceptibility to predators as an important mechanism [7,8], although only a few studies have explored this. The decrease in galler density after N deposition may be related to changes in plant volatiles that are essential for successful foraging and attacks by predators. Our understanding of this mechanism remains limited, and we strongly encourage future work on the effect of N deposition on the susceptibility of insect herbivores to predation.

5. Conclusions

Our large-scale N fertilization experiment is the first manipulative study on tall N₂-fixing trees and their insect herbivores. Fertilization has a positive, plant-mediated effect on leaf damage by chewers via a decrease in physical defense (LMA). Our study provides valuable information on large-scale N fertilization (i.e., N deposition) effects on insect–plant interactions in N₂-fixing plants, which may be helpful in predicting future climate change impacts.

Supplementary Materials: The following are available online at <https://www.mdpi.com/1999-4907/12/2/210/s1>, Table S1: List of herbivory species collected in this study.

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