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Ozone alters the feeding behavior of the leaf beetle *Agelastica coerulea* (Coleoptera: Chrysomelidae) into leaves of Japanese white birch (*Betula platyphylla* var. *japonica*)

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Abstract

High mixing ratios of ground-level O₃ threaten trophic interactions. In the present study, we conducted laboratory assays, where insect larvae and adults were not directly exposed to O₃, to test the feeding behavior and attraction of the coleopteran leaf beetle *Agelastica coerulea* to early and late leaves of Japanese white birch (*Betula platyphylla* var. *japonica*) treated with ambient or elevated O₃ levels. We found that overwintered adults were not deterred from grazing elevated O₃-treated leaves, but rather preferred them than ambient O₃-treated ones. We also found that the feeding behavior of 2nd instar larvae fed on early or late leaves was not influenced by the O₃ treatment of the leaves when larvae could choose leaves. These observations of the adults and larvae feeding preferences contradict prior observations in the field conditions where the insects avoided leaves in O₃-enriched atmosphere. Since adults preferred elevated O₃-exposed leaves in the present laboratory assays; it is worthy of further investigations whether adults change their grazing preference so as to ensure the leaf palatability as a feeding source for their larvae. Hence, new direction towards detailed ovipositional behavior surveys under field conditions is encouraged.

Keywords: adult, feeding, herbivory, insect grazing, larva, phytophagous insect, trophic interaction, tropospheric O₃

1. INTRODUCTION

Ground-level ozone (O₃) levels in the Northern Hemisphere have been continuously increasing after the Industrial Revolution (Akimoto 2003; Kalabokas et al. 2013; Saitanis et al. 2015; Kopanakis et al. 2016). High O₃ levels can cause toxicity to plants,

alter trophic interactions, lead to yield losses and impair ecosystems functioning and sustainability in the long term (Cape 2008; Feng et al. 2008; Lindroth 2010; Koike et al. 2013; Agathokleous et al. 2015, 2016). In particular, photosynthesis of plants is usually inhibited by elevated O₃ levels and consequently plant defense may be lowered, as defensive chemicals are mostly originated from carbon-based secondary compounds (Bryant et al. 1983; Jones and Colman 1988; Baur et al. 1991; Lindroth 2010; Karabourniotis et al. 2014; Agathokleous et al. 2016). In this framework, carbon: nutrient balance (CNB) hypothesis (Bryant et al. 1983) suggests that an increase of grazing damage by insect herbivores could be expected due to low defense capacity of leaf via photosynthetic suppression by O₃.

The coleopteran leaf beetle *Agelastica coerulea* (Baly, 1874) (hereafter leaf beetle) is an oligophagous herbivorous insect feeding on alder and birch (e.g. Baur et al. 1991; Sakikawa et al. 2016). Its life cycle has been described by Hara (2015): In Sapporo, northern Japan, adults of leaf beetle overwinter and start grazing early leaves of birch from May to early June. Yet, they start ovipositing eggs on the lower surface of the leaves and partly on well expanded late leaves rather than young leaves. After late June to early July, larvae of leaf beetle start grazing adaxial surface of leaves by late July to early August; larvae can go through only small distances. Larvae at the ultimate stage (prepupae) fall on the ground and start pupation in soil by mid-August, and the pupae exclude to adult emergence after about two weeks. The adults start grazing leaves again and move toward the ground for overwintering around late September.

Once leaves are grazed by insect herbivores, plant should shed them to avoid further grazing (e.g. Schoonhoven et al. 2002). Recently, summer leaf shedding after insect grazing of Japanese white birch saplings (*Betula platyphylla* var. *japonica*) occurred markedly in ambient O₃ but not at all in elevated O₃ atmosphere in Free Air Controlled Exposure (FACE) systems (Sakikawa et al. 2014). In contrast to expectations based on the low defense capacity theory; it has been observed that the beetle was deterred from grazing leaves of Japanese white birch exposed to O₃-enriched atmosphere in FACE systems (Sakikawa et al. 2014, 2016; Vanderstock et al. 2016).

In agreement with these findings, elevated O₃ caused reduction in insect abundance and alterations in community composition of an aspen-birch forest at the aspen CO₂ FACE site in Wisconsin, USA (Hillstrom and Lindroth 2008). These observations were done when both insects and plants were directly exposed to O₃. However, three questions evolve: 1) What is the feeding behavior of the beetle when it is not directly exposed to O₃? 2) Is the feeding behavior of overwintered adults and 2nd instar larvae (hereafter larvae) similar? 3) Does the type of leaf affect the insect feeding behavior?

The present research aims to study the feeding behavior of the beetle when it is not directly exposed to elevated O₃, within two contexts: 1) by a no-choice assay, where individual beetles could not make their choice between leaves treated with ambient O₃

or elevated O₃ levels, and, 2) by a choice assay, where individual beetles could make their choice between leaves treated with ambient O₃ and elevated O₃ levels.

As to the non-choice assay, we hypothesized that the beetle consumption would change upon O₃ treatment of early or late leaves, when adults or larvae have no option to select the feeding source. The fundamental of this hypothesis was an increased consumption of leaves by insects because of elevated O₃-induced reduced leaf palatability. Regarding the choice assay, we hypothesized that the beetle herbivory preference changes upon O₃ treatment of early or late leaves when overwintered adults or larvae have the option to select between ambient O₃-treated and elevated O₃-treated leaves. This hypothesis was based on insect attraction to elevated O₃-treated leaves as a result of elevated O₃-induced reduced leaf defense. As to the leaf type, we hypothesized that adults, which are chewers, would not be influenced by leaf types, whereas larvae, which are skeletonizers, would be influenced by leaf types, with preference towards early leaves because of lower mechanical strength than late leaves.

2. MATERIALS AND METHODS

2.1. Feeding assays

For the no-choice assay, a leaf and an overwintered adult beetle (hereafter adult) were placed in a cup (diameter = 8 cm, volume $\approx$ 280 cm³) which contained either an early or a late leaf of Japanese white birch (Koike 1995, Hoshika *et al.* 2012) obtained from ambient O₃ or elevated O₃ atmospheres (*i.e.* four conditions). The adult was fasted for twelve hours in the light and left there for twelve consecutive hours. Then, a fresh leaf was scanned (Canon LIDE 40, Tokyo) and placed in the cup. Twelve hours later, the grazed leaves were scanned (Canon LIDE 40, Tokyo) again. The leaf area was measured (free software LAI-32, Yamamoto, K) in both photos, and the grazed leaf area (mm²) was calculated by subtracting the final leaf area from the initial leaf area.

For the choice assay, six adults were placed in an empty experimental cage (18.5 × 16.5 × 8.0 cm) for twelve consecutive hours, and then, they were provided with early and late leaves exposed to ambient O₃ and elevated O₃ for twelve hours. After twelve hours of grazing (hypothetical), the remaining leaf area was estimated using images of scanned leaves, as described above. Identical no-choice and choice assays were conducted with individual larvae, but fasted for six hours rather than twelve hours and each was placed in an 8-cm cup. After the fasting period, each larva was supplied with a 12 mm leaf disk because the movement distance of larvae is comparatively short..

The assays with adults were conducted in late May to early June, whereas the assays with larvae were conducted in July, in a laboratory incubator (MIR-254-PJ, Panasonic, Tokyo) with a constant temperature of 25 °C and PFD $\approx$ 500 μ mol m⁻²s⁻¹, and replicated six times. For both assays, larvae and adults were collected from the Sapporo

Experimental Forest of Hokkaido University (43°06' N, 141°20' E), in 2014. The leaves were collected from twelve Japanese white birch saplings planted in experimental sites (20th April 2011, 4-year old at planting time) located within the same experimental forest and exposed to either ambient or elevated O₃ atmosphere. For the elevated O₃ treatment, the target O₃ level was 60 nmol mol⁻¹ during daylight hours. The saplings were exposed to O₃ treatments in the growing seasons 2011-2014. In 2014, when the feeding assays were conducted, the O₃ treatments lasted from May to November, and the daytime hourly mean O₃ levels in ambient and elevated O₃ were 27.5 ±11.6 nmol mol⁻¹ and 61.5 ±13.0 nmol mol⁻¹. Height and diameter at breast height in ambient and elevated O₃ sites were 6.2 (±0.5) and 3.8 (±0.3) cm and 6.0 (±0.7) and 3.2 (±0.4), respectively, in mid-September, 2015. More information on the free-air O₃ enrichment system and trees can be found in Watanabe *et al.* (2013) and Sakikawa *et al.* (2016).

2.2. Leaf phenolic analyses and leaf strength measurements

Measurements of defensive traits were carried out in late July 2014 using six samples per O₃ treatment. The third and fourth leaves, counted from shoot base, were sampled from two different shoots per sapling to adjust leaf age and physiological function (Koike 1995). The sampled leaves were immediately freeze-dried (FLEXI-DRY, FTS Systems, Stony Ridge NY, USA) and mill-grounded (TM10, Tescom, Tokyo, Japan) to powder. The four leaves collected from each sapling were mingled to provide one robust sample per sapling for measurement.

The content of total phenolics in 20 mg of leaf powder was determined following the Folin–Ciocalteu method, as modified by Julkunen-Titto (1985). The content of condensed tannin was determined following the proanthocyanidin method (Bate-Smith 1977; Matsuki *et al.* 2004). Leaf disks (diameter = 8 mm) were punched from the center part of the leaf blade (avoiding the main vein) and were dried in an oven at 60 °C for two consecutive days. Dry masses of leaf disks were then measured to determine the leaf mass per unit area (LMA: g cm⁻²).

2.3. Statistics

The data of grazed leaf area, LMA, phenolics and tannin were transformed by a Box-Cox transformation (Box and Cox 1964) for a better approximation to the Gaussian distribution. The hypothesis of feeding assays was tested by a General Linear Model, for each insect stage (*i.e.* adults or larvae) and each assay (*i.e.* choice and no-choice), with fixed factors the O₃ treatment, from which the leaf was obtained from (ambient or elevated), and the leaf type (*i.e.* early or late). For multiple comparisons among three or more groups, a Bonferroni post-hoc test followed. The hypothesis of LMA and the content of phenolics and tannin was tested by two-way analysis of variance (ANOVA). All the analyses were done using six replicates per experimental condition. The alpha level was predefined at 0.05. Data handling and statistical analyses were performed

using MS EXCEL 2010 (Microsoft®) and STATISTICA v.10 (StatSoft® Inc.).

3. RESULTS & DISCUSSION

Based on the observations, adults and larvae started to graze leaves only after tasting all the available leaves. As to the no-choice assay with overwintered adults, leaves obtained from elevated O₃ had about 1.5 times higher grazed area than those obtained from ambient O₃, however the difference was statistically non-significant (Fig 1, upper). On the other hand, in the choice assay, leaves obtained from elevated O₃ had about 6 times greater grazed area than those obtained from ambient O₃ (Fig 1, lower). These results support the low-defense-based prediction and are in line with the findings of Freiwald *et al.* (2008), where female adults of the beetle *Phyllobius pyri* (Linnaeus, 1758) preferred and consumed more elevated O₃-exposed leaves of two hybrid aspen clones (*Populus tremula* L. × *P. tremuloides* Michx.). Leaf type and O₃ × Leaf type were insignificant factors for both choice and no-choice assay.

Larvae (Fig 2) grazed only interveinal mesophyll areas (photos in Vanderstock *et al.* (2016)) and showed a different feeding behavior from adults: In the no-choice assay (Fig 2, upper), larvae grazed 1.6 times more early leaves obtained from elevated O₃ than early leaves obtained from ambient O₃, however the difference was statistically non-significant, and 3.5 times less late leaves obtained from elevated O₃ than late leaves obtained from ambient O₃. Independently from the O₃ treatment of leaves, larvae fed with 3.3 times more early than late leaves; this finding shows that larvae consumption was changed via minimized feeding on late leaves, suggesting that late leaves were not attractive to the larvae. Similarly to the no-choice assay, in the choice assay (Fig 2, lower), larvae grazed 7-fold more early leaves than late leaves, and their feeding preference was not affected by O₃ treatment of leaves. Agrell *et al.* (2005) found that larvae of the *Malacosoma disstria* (Hübner 1820) (Lepidoptera: Lasiocampidae) fed with more ambient O₃-treated than elevated O₃-treated leaves of two aspen genotypes (*Populus tremuloides* Michx.) but did not show any preference between ambient and elevated O₃-treated leaves of birch (*Betula papyrifera* Marsh.). In the present assays, larvae (Fig 2) did not show preference towards ambient O₃ leaves, and only in one out of four cases grazed greater leaf area compared to elevated O₃.

Tannins are the most abundant secondary metabolites produced by plants and along with phenolics protect plants against herbivores (Barbehenn and Constabel 2011; Karabourniotis *et al.* 2014). LMA is also positively correlated with leaf mechanical strength (Koike *et al.* 2006). No difference was found in total phenolics and condensed tannin between early and late leaves, whereas the LMA of early leaves was lower than that of late leaves (Fig 3). This LMA finding verifies that changes in larvae feeding preference were upon leaf thickness. However, LMA did not differ between ambient and elevated O₃-exposed leaves, showing that leaf thickness had no role in the changed feeding preference of adults. This phenomenon of different feeding behavior between

larvae and adults in relation to LMA can be attributed to the grazing characteristics of them: chewing by adults vs. skeletonizing of leaf mesophyll by larvae (Bauer *et al.* 1991). White birch, which is an early successional species, is expected to compensate for stress or disturbance via growth at the expense of defense. Ambient O₃ leaves had greater content of total phenolics (+13.2 mg g⁻¹, 125%) and condensed tannin (+3.7 mg g⁻¹, 147%) than elevated O₃ leaves. This finding is consistent with the expectations based on the CNB hypothesis and indicates reduced defense capacity of elevated O₃ leaves. Reduction of total phenolic and tannin contents has been also found in *Tibouchina pulchra* (Cham.) Cogn. saplings exposed to elevated O₃ (Da Rosa Santos and Furlan 2013). However, in other studies with C₃ plants, it has been found that condensed tannin (Jordan *et al.* 1991; Peltonen *et al.* 2005; De Rezende and Furlan 2009) and phenolics (Yamaji *et al.* 2003; Fares *et al.* 2010; Booker *et al.* 2012) were positively affected by elevated O₃.

In other cases, total phenolic content or concentration remained unaffected whereas tannins were affected by elevated O₃ (Jordan *et al.* 1991) or different phenols showed different patterns (increase or decrease) in elevated O₃ (Kanoun *et al.* 2001). It can be suggested that the impacts of elevated O₃ can be qualitative (i.e. altered allocation within the phenolics) rather than quantitative (i.e. increase in total content) (Jordan *et al.* 1991; Kanoun *et al.* 2001; Yamaji *et al.* 2003; Peltonen *et al.* 2005; Fares *et al.* 2010). Nonetheless, these secondary metabolites hold a major role in the strategies of plants for carbon gain or water saving and growth or defense, and thus their abundance depends on the environment, such as between dry and wet climates (Karabourniotis *et al.* 2014).

Leaf consumption by insects may increase, decrease or remain unaffected under elevated O₃, and it seems that the outcome varies upon plant species and insect stage (Jones and Coleman 1988; Endress *et al.* 1991; Bolsinger *et al.* 1992; Agrell *et al.* 2005; Freiwald *et al.* 2008). The present results of the no-choice assays indicate that both larvae and adults did not need to significantly increase consumption in order to compensate for significant O₃-induced reductions in leaf palatability. In general, when herbivore insects feed with plant tissues with lower nitrogen concentration they increase consumption to compensate for the nitrogen acquisition. It is therefore speculated that ambient and elevated O₃-treated leaves might not differ in nitrogen content. Furthermore, these results do not support suggestions by Bolsinger *et al.* (1992) that the primary cause of altered behavior and performance may be the O₃-induced stimulation of insect feeding.

The present findings contradict the previous unexplained observations in the field, where the beetle grazing was reduced in elevated O₃ (Sakikawa *et al.* 2014, 2016; Vanderstock *et al.* 2016). The present insect assays provide evidence that leaf palatability was not the primary reason for the field observations. Hence, other potential reasons should be considered.

In field conditions, the grazing of the adult beetle was similar between ambient and elevated O₃ in the first week of observation (early spring), but it remained low in elevated O₃ (along with larvae grazing) and continued to increase in ambient O₃ from second week and onwards (Vanderstock *et al.* 2016). Fuentes *et al.* (2013), in a laboratory experiment with insects and source of volatiles at a close distance, showed that the striped cucumber beetle (*Acalymma vittatum*, Fabricius 1775) (Coleoptera: Chrysomelidae) did not avoid O₃ polluted air (mixing ratios up to 120 nmol mol⁻¹) when it had the choice to select between clean and polluted air. This finding may indicate that the beetle did not perceive O₃ as harmful chemical and suggest excluding the possibility of O₃ direct effects on beetle grazing preference.

Another reason, which requires further experimentation, is a potential O₃-induced alteration of plant-emitted biogenic volatile organic compounds (VOCs) which can occur either indirectly, through O₃-induced biological alterations in plants, or directly, by reaction of O₃ with VOCs in the atmosphere which results in VOC breakdown products (Fuhrer and Booker 2003; Lindroth *et al.* 2010; Blande *et al.* 2014). Plants emit VOCs in the framework of a defense system that permits plant-to-plant communication; these VOCs act as a signal for insects and, thus, hold an important role in plant-insect interactions in a wide variety of vegetation types (Blande *et al.* 2014). It has been recently shown that degradation of floral scent and chemical transformations in the scent blend imbed herbivore ability to trace its host (Fuentes *et al.* 2013; Farré-Armengol *et al.* 2016). Furthermore, it has been shown that, under the influence of O₃, herbivore-attracting leaf VOCs may become degraded to chemical compounds, which on leaf surface can act as a repellent for leaf-feeding herbivores (Li *et al.* 2016). If herbivore-attracting leaf VOCs become degraded and act as a repellent for insects in elevated O₃ (Fuentes *et al.* 2013; Farré-Armengol *et al.* 2016; Li *et al.* 2016) while reactive VOC emissions increase in ambient O₃ because of greater foliage damage caused by insect herbivory (Yli-Pirilä *et al.* 2016), the insects are likely to move into ambient O₃ condition than elevated O₃ condition in a FACE system. However, in our laboratory assays herbivores not only were not deterred from grazing O₃-injured leaves but also consumed more tissue of leaves obtained from elevated O₃ than of leaves obtained ambient O₃.

The final, and most likely, explanation for the field observations is insect oviposition: Fewer eggs and larvae of the beetle were found in elevated O₃ than ambient O₃ (Inoue *et al.* 2016). As in the present laboratory assays insects were not deterred from grazing O₃-injured leaves, it may be postulated that, after a time, adults may avoid ovipositing on O₃-injured leaves in order to ensure the food quality for the offspring (Schoonhoven *et al.* 2012). In this framework, further studies on insect ecology and physiology are needed.

4. CONCLUSIONS

- (a) In agreement with the initial hypothesis, when the privilege of leaf choice was given to insects, the feeding behavior of the adults changed upon O₃ treatment of leaves, whereas the feeding behavior of the larvae changed upon leaf type but not upon O₃ treatment;
- (b) when insects could not choose among leaves, the leaf consumption of larvae changed, as it was mainly evidenced by reduced grazing of late leaves; however, the consumption of adults remained unchanged.
- (c) altered feeding behavior and consumption of the leaf beetle was not upon O₃-induced stimulation of insect feeding as a result of reduced leaf palatability;
- (d) decreased palatability of O₃-exposed, white birch leaves was not the primary cause for reduction of the leaf beetle herbivory in an O₃-polluted atmosphere;
- (e) detailed surveys of insect oviposition, individual and in aggregation, are needed in order to test the hypothesis whether adult insects change their behavior in a framework of a strategy to preserve leaf palatability for the offspring.

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Figures & Captions

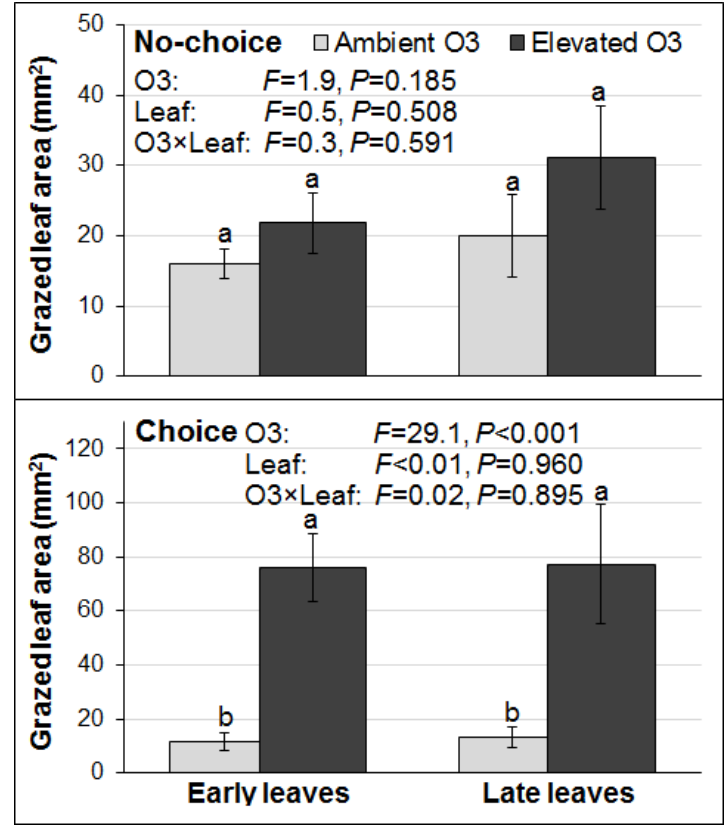


Fig 1. Mean values $\pm$ s.e. ($n = 6$) of grazed leaf area in no-choice and choice feeding assays of overwintered adults of the coleopterous leaf beetle *Agelastica coerulea* Baly with early and late leaves of Japanese white birch (*Betula platyphylla* var. *japonica*) saplings exposed to ambient O₃ or O₃-enriched atmospheres. Statistical analysis was performed with General Linear Model, and, when needed, followed by Bonferroni post-hoc test. Different letters above the standard error bars of the means indicate statistically significant difference at an $\alpha = 0.05$.

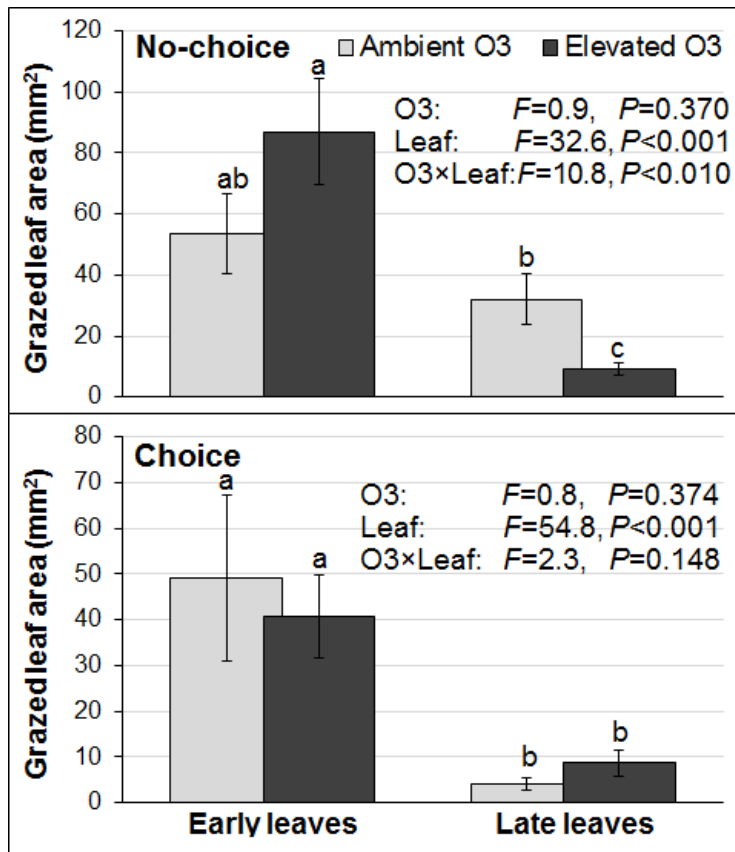


Fig 2. Mean values $\pm$ s.e. ($n = 6$) of grazed leaf area in no-choice and choice feeding assays of 2nd instar larvae of the coleopteran leaf beetle *Agelastica coerulea* (Baly, 1874) fed with early and late leaves of Japanese white birch (*Betula platyphylla* var. *japonica*) saplings exposed to ambient O₃ or O₃-enriched atmospheres. Statistical analysis was performed with General Linear Model and, when needed, followed by Bonferroni post-hoc test. Different letters above the standard error bars of the means indicate statistically significant difference at an $\alpha = 0.05$.

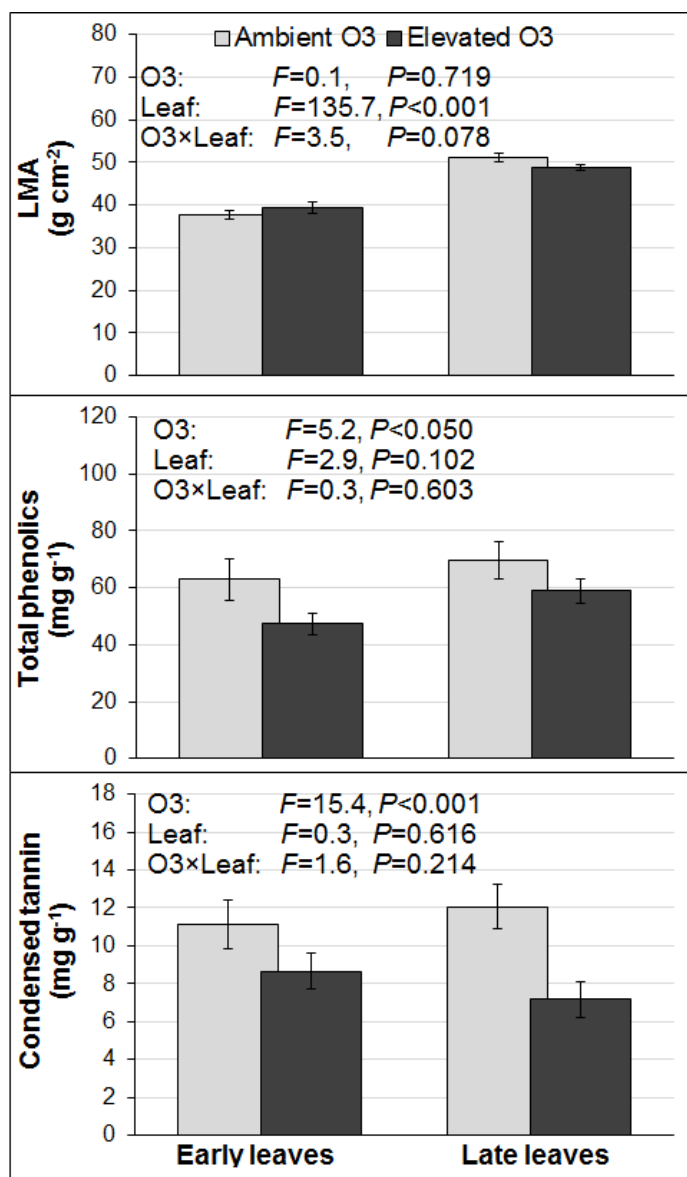


Fig 3. Mean values $\pm$ s.e. ($n = 6$) of leaf mass per area (LMA) and content of total phenolics and condensed tannin of early and late leaves in ambient and elevated O₃ atmospheres. Statistical analysis was performed with two-way ANOVA at an $\alpha = 0.05$.