



Title	Ozone alters the feeding behavior of the leaf beetle <i>Agelastica coerulea</i> (Coleoptera: Chrysomelidae) into leaves of Japanese white birch (<i>Betula platyphylla</i> var. <i>japonica</i>)
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43 alter trophic interactions, lead to yield losses and impair ecosystems functioning and
44 sustainability in the long term (Cape 2008; Feng et al. 2008; Lindroth 2010; Koike et al.
45 2013; Agathokleous et al. 2015, 2016). In particular, photosynthesis of plants is usually
46 inhibited by elevated O₃ levels and consequently plant defense may be lowered, as
47 defensive chemicals are mostly originated from carbon-based secondary compounds
48 (Bryant et al. 1983; Jones and Colman 1988; Baur et al. 1991; Lindroth 2010;
49 Karabourniotis et al. 2014; Agathokleous et al. 2016). In this framework, carbon:
50 nutrient balance (CNB) hypothesis (Bryant et al. 1983) suggests that an increase of
51 grazing damage by insect herbivores could be expected due to low defense capacity of
52 leaf via photosynthetic suppression by O₃.

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

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

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

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

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

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

96

2. MATERIALS AND METHODS

97 2.1. Feeding assays

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

107 For the choice assay, six adults were placed in an empty experimental cage (18.5 \times 16.5
108 \times 8.0 cm) for twelve consecutive hours, and then, they were provided with early and
109 late leaves exposed to ambient O₃ and elevated O₃ for twelve hours. After twelve hours
110 of grazing (hypothetical), the remaining leaf area was estimated using images of
111 scanned leaves, as described above. Identical no-choice and choice assays were
112 conducted with individual larvae, but fasted for six hours rather than twelve hours and
113 each was placed in an 8-cm cup. After the fasting period, each larva was supplied with a
114 12 mm leaf disk because the movement distance of larvae is comparatively short..

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

119 Experimental Forest of Hokkaido University (43°06' N, 141°20' E), in 2014. The leaves
120 were collected from twelve Japanese white birch saplings planted in experimental sites
121 (20th April 2011, 4-year old at planting time) located within the same experimental
122 forest and exposed to either ambient or elevated O₃ atmosphere. For the elevated O₃
123 treatment, the target O₃ level was 60 nmol mol⁻¹ during daylight hours. The saplings
124 were exposed to O₃ treatments in the growing seasons 2011-2014. In 2014, when the
125 feeding assays were conducted, the O₃ treatments lasted from May to November, and
126 the daytime hourly mean O₃ levels in ambient and elevated O₃ were 27.5 ±11.6 nmol
127 mol⁻¹ and 61.5 ±13.0 nmol mol⁻¹. Height and diameter at breast height in ambient and
128 elevated O₃ sites were 6.2 (±0.5) and 3.8 (±0.3) cm and 6.0 (±0.7) and 3.2 (±0.4),
129 respectively, in mid-September, 2015. More information on the free-air O₃ enrichment
130 system and trees can be found in Watanabe *et al.* (2013) and Sakikawa *et al.* (2016).

131 2.2. Leaf phenolic analyses and leaf strength measurements

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

139 The content of total phenolics in 20 mg of leaf powder was determined following the
140 Folin–Ciocalteu method, as modified by Julkunen-Titto (1985). The content of
141 condensed tannin was determined following the proanthocyanidin method (Bate-Smith
142 1977; Matsuki *et al.* 2004). Leaf disks (diameter = 8 mm) were punched from the center
143 part of the leaf blade (avoiding the main vein) and were dried in an oven at 60 °C for
144 two consecutive days. Dry masses of leaf disks were then measured to determine the
145 leaf mass per unit area (LMA: g cm⁻²).

146 2.3. Statistics

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

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

158

3. RESULTS & DISCUSSION

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

170 Larvae (Fig 2) grazed only interveinal mesophyll areas (photos in Vanderstock *et al.*
171 (2016)) and showed a different feeding behavior from adults: In the no-choice assay
172 (Fig 2, upper), larvae grazed 1.6 times more early leaves obtained from elevated O₃ than
173 early leaves obtained from ambient O₃, however the difference was statistically
174 non-significant, and 3.5 times less late leaves obtained from elevated O₃ than late leaves
175 obtained from ambient O₃. Independently from the O₃ treatment of leaves, larvae fed
176 with 3.3 times more early than late leaves; this finding shows that larvae consumption
177 was changed via minimized feeding on late leaves, suggesting that late leaves were not
178 attractive to the larvae. Similarly to the no-choice assay, in the choice assay (Fig 2,
179 lower), larvae grazed 7-fold more early leaves than late leaves, and their feeding
180 preference was not affected by O₃ treatment of leaves. Agrell *et al.* (2005) found that
181 larvae of the *Malacosoma disstria* (Hübner 1820) (Lepidoptera: Lasiocampidae) fed
182 with more ambient O₃-treated than elevated O₃-treated leaves of two aspen genotypes
183 (*Populus tremuloides* Michx.) but did not show any preference between ambient and
184 elevated O₃-treated leaves of birch (*Betula papyrifera* Marsh.). In the present assays,
185 larvae (Fig 2) did not show preference towards ambient O₃ leaves, and only in one out
186 of four cases grazed greater leaf area compared to elevated O₃.

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

196 larvae and adults in relation to LMA can be attributed to the grazing characteristics of
197 them: chewing by adults vs. skeletonizing of leaf mesophyll by larvae (Bauer *et al.*
198 1991). White birch, which is an early successional species, is expected to compensate
199 for stress or disturbance via growth at the expense of defense. Ambient O₃ leaves had
200 greater content of total phenolics (+13.2 mg g⁻¹, 125%) and condensed tannin (+3.7 mg
201 g⁻¹, 147%) than elevated O₃ leaves. This finding is consistent with the expectations
202 based on the CNB hypothesis and indicates reduced defense capacity of elevated O₃
203 leaves. Reduction of total phenolic and tannin contents has been also found in
204 *Tibouchina pulchra* (Cham.) Cogn. saplings exposed to elevated O₃ (Da Rosa Santos
205 and Furlan 2013). However, in other studies with C₃ plants, it has been found that
206 condensed tannin (Jordan *et al.* 1991; Peltonen *et al.* 2005; De Rezende and Furlan
207 2009) and phenolics (Yamaji *et al.* 2003; Fares *et al.* 2010; Booker *et al.* 2012) were
208 positively affected by elevated O₃.

209 In other cases, total phenolic content or concentration remained unaffected whereas
210 tannins were affected by elevated O₃ (Jordan *et al.* 1991) or different phenols showed
211 different patterns (increase or decrease) in elevated O₃ (Kanoun *et al.* 2001). It can be
212 suggested that the impacts of elevated O₃ can be qualitative (i.e. altered allocation
213 within the phenolics) rather than quantitative (i.e. increase in total content) (Jordan *et al.*
214 1991; Kanoun *et al.* 2001; Yamaji *et al.* 2003; Peltonen *et al.* 2005; Fares *et al.* 2010).
215 Nonetheless, these secondary metabolites hold a major role in the strategies of plants for
216 carbon gain or water saving and growth or defense, and thus their abundance depends
217 on the environment, such as between dry and wet climates (Karabourniotis *et al.* 2014).

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

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

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

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

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

273

4. CONCLUSIONS

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

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299

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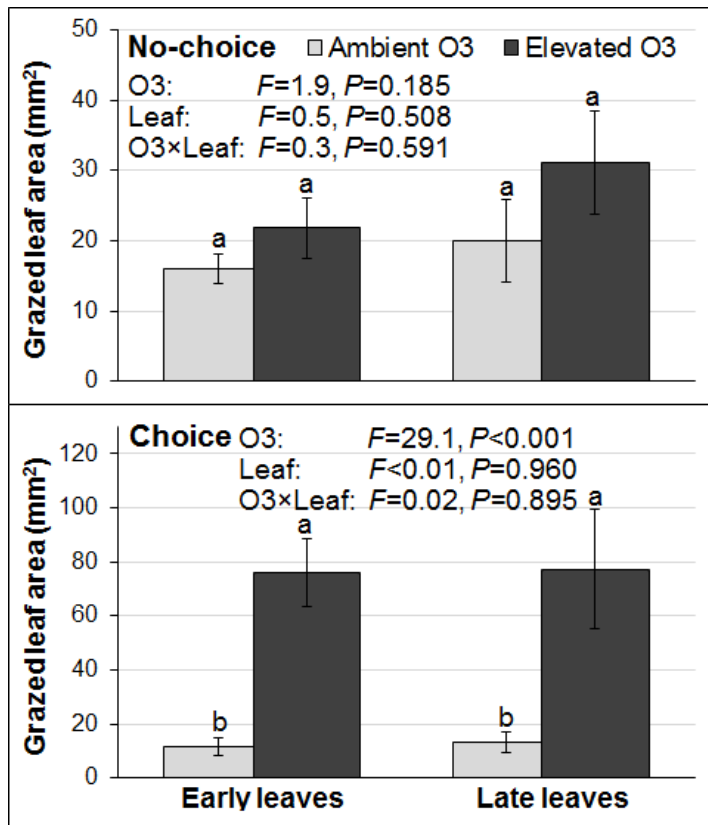
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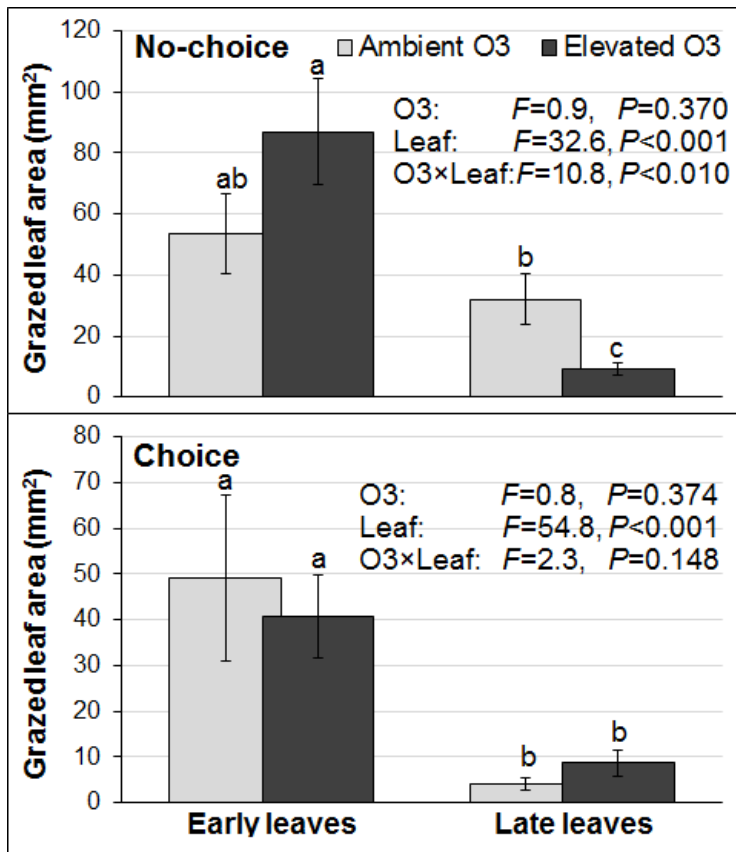
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Figures & Captions



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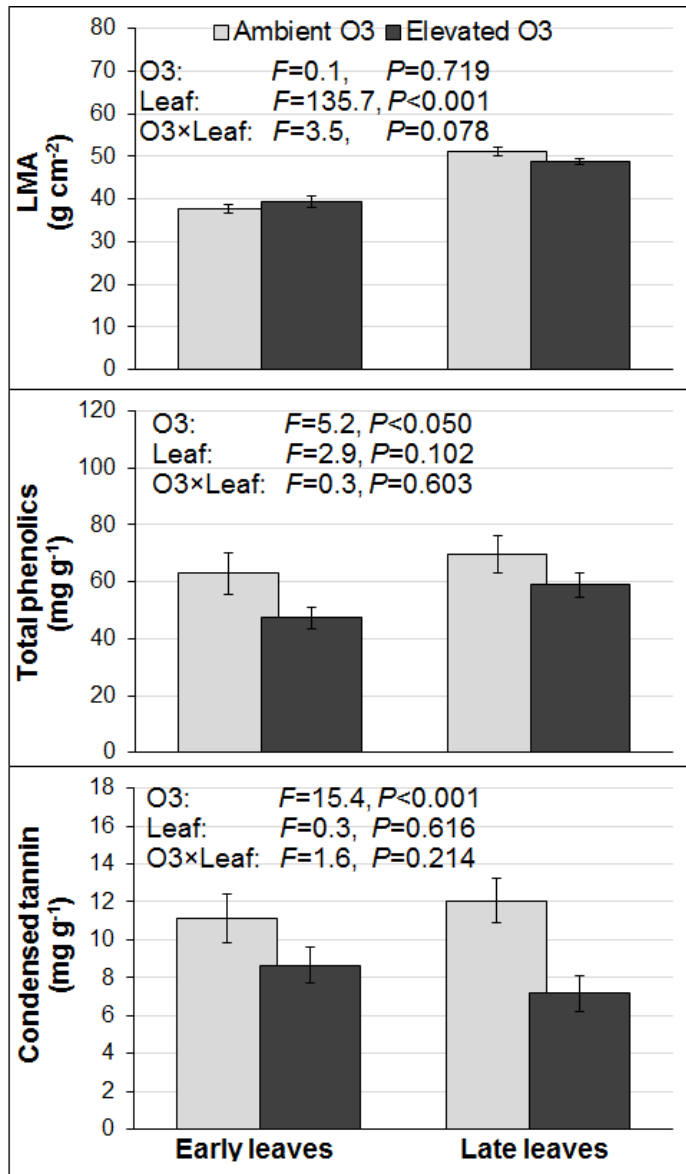
Fig 1. Mean values \pm s.e. ($n = 6$) of grazed leaf area in no-choice and choice feeding assays of overwintered adults of the coleopterous leaf beetle *Agelastica coerulea* Baly with early and late leaves of Japanese white birch (*Betula platyphylla* var. *japonica*) saplings exposed to ambient O₃ or 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 $\alpha = 0.05$.



456

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

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465

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