Growth and nutrition of *Agelastica coerulea* (Coleoptera: Chrysomelidae) larvae changed when fed with leaves obtained from an O_3–enriched atmosphere

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Abstract

A series of laboratory no-choice assays were performed to test changes in the feeding, growth and nutrition of leaf beetle (*Agelastica coerulea*) larval instars on O_3-treated leaves of Japanese white birch (*Betula platyphylla* var. *japonica*). Larvae fed with O_3-treated leaves grew and developed significantly faster throughout their developmental cycle than the corresponding controls. The growth rate (GR) and consumption index (CI) were mostly decreased with age for both control and O_3-treated leaves. Efficiency of

conversion of both ingested and digested food (ECI, ECD) showed an increase from the 2nd to the 4th instar, after which they decreased significantly and reached the lowest value in the last larval instars (7th). GR, CI, ECI and ECD were greater and approximate digestibility (AD) lower in larvae fed with O_3-treated leaves than those fed with control leaves. This indicated that the greater rate of growth on fumigated leaves was due primarily to a greater rate of consumption (*i.e.*, O_3 increased the "acceptability" of the host more than "suitability") and efficiency in converting food into body mass. Overall, larval performance seemed to have improved when fed with O_3-treated leaves in these assays. This study suggests insects may be more injurious to O_3-treated plants and warrants further investigations on birch-beetle interactions under field conditions.

Keywords: air pollution; herbivore; leaf beetle; nutrition; plant-insect; trophic interaction

1. INTRODUCTION

Ground-level ozone (O_3) concentrations in the lower troposphere have notably increased since the preindustrial age, and remain at potentially phytotoxic levels, although control measures on precursor emissions have reduced O_3 peaks in some regions of Europe (Vingarzan 2004; Derwent et al. 2007; Cape 2008; The Royal Society 2008; Sicard et al.

47 2016, 2017; Solomou et al. 2018). Northeast Asia is a hot spot of O_3 pollution
48 (Nagashima et al. 2017; Trieu et al. 2017; Wang et al. 2017). Domestically-produced O_3
49 and transboundary transport from outside, mainly from China, result in significant
50 increases in O_3 levels in Japan in recent years (Nagashima et al. 2010, 2017; Tanimoto
51 2009; Tanimoto et al. 2009). Furthermore, an increase in background O_3 levels in regions
52 of the northern hemisphere may occur in the near future (The Royal Society 2008). O_3 is
53 a gaseous pollutant which causes toxicity to plants at macro- and microscopic levels,
54 when its concentrations exceed species-specific thresholds, through reactions in the
55 leaves, with a subsequent production of highly oxidative intermediates (Robinson and
56 Rowland 1996; Oksanen et al. 2013; Vaultier and Jolivet 2015). Such exceedances may
57 result in reduction in plant vigor, suppression of yields and productivity, with further
58 implications to ecological processes and trophic cascades (Feng et al. 2008; Lindroth
59 2010; Ainsworth et al. 2012; Koike et al. 2013; Blande et al. 2014; Agathokleous et al.
60 2016a; Chappelka and Grulke 2016).
61 There has been a notable progress in research on O_3 effects on vegetation and plant
62 ecosystems over the last decades (Feng et al. 2008; Lindroth 2010; Ainsworth et al. 2012;
63 Koike et al. 2013; Blande et al. 2014; Agathokleous et al. 2015a,b, 2016); however, the
64 knowledge about O_3 effects on trophic interactions remains limited. The relationship

between plant stress and susceptibility to insects is of particular interest (White 1974, 1976, 1984; Alstad et al. 1982; Hain 1987; Mattson and Haack 1987). Plant-herbivore relationships represent a dynamic equilibrium between plant defense against herbivory and insect feeding adaptation on host plants. However, environmental stresses like air pollution can shift the balance of these relationships (Hughes 1988; Hillstrom and Lindroth 2008; Lindroth 2010; Chappelka and Grulke 2016; Agathokleous 2018). Stressed plants could become more vulnerable to herbivory when biochemical changes lead to an increase in the nutritional value or to a decrease in plant chemical defenses (White 1974, 1984; Valkama et al. 2007; Ali and Agrawal 2012; Chappelka and Grulke 2016). Stressed trees may be a more suitable food source for invertebrate herbivores than unstressed trees due to an increase in the tissue content of soluble nitrogenous compounds (Fred 1987; Koike et al. 2006). Although O_3 is known to change the palatability of leaves, how this change influences plant-insect interactions remains underexplored.

The insect performance depends on the level of toxins produced by plants and the quality of the insect, e.g. sequestering or stealthy (Ali and Agrawal 2012; Agathokleous 2018). In the case of O_3, the level of defense chemicals produced by plants, and thereby the insect performance, depend on the exposure level or on the O_3 dose uptake by plants, within the framework of hormesis (Agathokleous 2018). For instance, insects may display a

preference towards leaves which experience short exposure to elevated O_3 levels and have increased palatability (Jones and Coleman 1988a; Bolsinger et al. 1991, 1992). Recently, results from laboratory assays showed that O_3 altered the feeding behavior of the leaf beetle *Agelastica coerulea* (Baly 1874) (hereafter leaf beetle) into leaves of Japanese white birch (*Betula platyphylla* var. *japonica*) (Agathokleous et al. 2017a). In these assays, it was found that overwintered adults preferred elevated O_3-treated leaves than ambient O_3-treated ones and that the feeding behavior of 2nd instar larvae was not changed when larvae could select between ambient O_3-treated and elevated O_3-treated leaves (Agathokleous et al. 2017a). These laboratory observations are in agreement with observations in assays with the common leaf weevil *Phyllobius pyri* L. (Coleoptera: Curculionidae) (Freiwald et al. 2008) and oppose earlier field observations where the leaf beetle deterred from grazing leaves of Japanese white birch in elevated O_3 sites of a Free Air Controlled Exposure (FACE) system (Sakikawa et al. 2014, 2016; Vanderstock et al. 2016), a phenomenon which may be upon O_3-induced degradation of biogenic volatile organic compounds (BVOCs) that repels insects (Fuentes et al. 2013; Dötterl et al. 2016; Farré-Armengol et al. 2016; Li et al. 2016). However, in O_3-polluted regions, herbivore insects have no privilege to choose between O_3 sites, and, thus, feed on plants under stress. The implications of this feeding to the insect nutrition remain unknown.

101 In the present study, we explored the possibility that O_3 does affect insect nutrition
102 indirectly, when insects consume leaves which underwent elevated O_3 exposure. In order
103 to identify and quantify O_3 effects on insect nutrition, we conducted laboratory assays
104 with a collection of larvae instars of the leaf beetle fed with Japanese white birch leaves
105 obtained from sites with either background or elevated O_3 levels in a FACE system. We
106 hypothesized that O_3 would have indirect effects on the nutrition of larvae feeding with
107 O_3-treated leaves, and the effects could vary among larval instars which differ in their
108 anatomical and physiological characteristics.
109 We assessed larvae consumption, mass growth, efficiency of conversion of ingested food
110 (growth efficiency, ECI), efficiency of conversion of digested food (ECD), and
111 assimilation efficiency (approximate digestibility, AD) as effective indicators of food
112 utilization by insects (Slansky 1985). ECI relates the total consumption (food ingested) of
113 insect to the amount of body mass, whereas ECD ignores undigested food (Slansky 1985;
114 Farrar et al. 1989). AD indicates the food digestibility whereas ECI and ECD indicate the
115 insect efficiency to convert food into body mass.
116

2. MATERIALS AND METHODS

2.1. Insect eggs and leaf samples

Samples were collected in 2016 from Japanese white birch trees grown in the FACE system located at Sapporo Experimental Forest of Hokkaido University, Japan (43°04' N, 141°20' E, 15 m a.s.l.). This birch is classified as heterophyllous shoot development type with two types of leaves, namely early leaves vs. late leaves which appear after complete expansion of the early leaves (Koike 1995; Matsuki et al. 2004). These trees were planted in the experimental plots in mid-May 2014, when they were two-year old. The plants were periodically irrigated and treated with 100 times diluted wood vinegar early after plantation in 2014 for pest management until their establishment to the experimental sites. Fertilization was never carried out. After the establishment to the sites, plants were grown naturally with no intentional irrigation or other pest management. The snow cover period in Sapporo lasted from mid-December to early-May. Meteorological data in 2016 were recorded at a station located in Sapporo (WMO, ID: 47412), at 43^{o}03.6'N 141^{o}19.7'E (Japan Meteorological Agency 2017). For the period May-August, the main meteorological conditions (mean ± s.e.) were: mean monthly average of air temperature = 18.95 (±2.06) ^{o}C; daily maximum temperature = 23.48 (±1.95) ^{o}C; daily minimum temperature = 15.50 (±2.18) ^{o}C; wind speed = 4.00 (±0.23) m s^{-1}; relative humidity =

69.75 (±3.94) %; mean monthly total sunshine duration = 205.90 (±16.54) h; and mean monthly precipitation 137.63 (±52.36) mm, respectively. Meteorological data for the years 2014 and 2015 along with details of the study site are available in Agathokleous et al. (2017b).

The O_3-FACE system consisted of six rings; three with ambient air and three with ambient air enriched with O_3 (target = 70 nmol mol^{-1}) during daylight hours with photosynthetic photon flux density (PPFD) > 70 μmol m^{-2} s^{-1} (07:00–17:00). In 2014, the plants were exposed to elevated O_3 from August 15 to October 26, and, in 2015, from April 24 to October 26. The mean O_3 levels (07:00–17:00) for elevated O_3 plots were 60 nmol mol^{-1} in 2014 and 72 nmol mol^{-1} in 2015, whereas for ambient O_3 plots were 20 nmol mol^{-1} in 2014 and 34 nmol mol^{-1} in 2015. In 2016, the O_3 enrichment started on May 18, and the last leaf samples were collected on July 15. The mean O_3 levels (07:00–17:00) in elevated O_3 plots were 63.52 nmol mol^{-1} during the period May 10 to July 15 and 60.92 nmol mol^{-1} during the period June 21 to July 15 (duration of insect assays). Ambient O_3 levels were recorded from June 1 onwards. Ambient O_3 levels (07:00–17:00) were 5.21 and 5.84 nmol mol^{-1} during the periods June 1 to July 15 and June 21 to July 15. Details on the FACE system and O_3 exposures can be found in Agathokleous et al. (2017b).

153 **2.2. Laboratory insect culture and assays**

154 The colony of the leaf beetle was started with egg patches obtained from ambient air
155 conditions of Sapporo Experimental Forest of Hokkaido University, on June 16, 2016.
156 Deposited eggs were selected based on the intensity of yellow color (eggs close to
157 hatching avoided) and transferred to laboratory. Thereafter, all the processes were
158 conducted under laboratory conditions. Eggs hatching occurred within four to five days
159 from the collection. Newly hatched larvae were placed in plastic boxes (12 × 6 × 18 cm).
160 Ten larvae were placed in a box replicated four times per O_3 treatment per instar stage,
161 giving thus a total of 40 individual larvae per instar per O_3 treatment. The assays were
162 conducted based on a completely randomized design, and the position of the boxes was
163 rotated on a daily base. The initial weight of the larvae was measured and the culture was
164 kept at a temperature of 25 ± 2°C, 65 ± 5 % relative humidity and 14:10 hours (D: L)
165 photoperiod (Abu ElEla and ElSayed 2015; Adham et al 2005 a,b; Abu ElEla et al 2016).
166 Larvae of each preceding instar molted and transferred to next stage by shedding the skin.
167 Larvae were fed on weighed quantities of treated and untreated fresh mature leaves of
168 Japanese white birch leaves until pupation. Fresh late leaves were collected from ambient
169 and elevated O_3 plots and provided to the larvae on a daily base, until the end of larval
170 stage. Late leaves were selected over early leaves because females of this beetle

commonly oviposit on late leaves and from June larvae start hatching and grazing these leaves (Agathokleous et al. 2017a). The leaves were randomly selected; however leaves with injury were excluded to minimize potential bias. To overcome limitations which may occur when using leaf disks, intact leaves were used (Jones and Coleman 1988b; Smith et al. 1994; Lacey 1997). Plant materials of each O_3 treatment were daily pooled, and leaf samples were randomly drawn and provided to the larvae. Feeding period lasted from June 21 to July 15.

In the middle of the assays, additional leaf samples, similar to those used for the assays, were collected from each plot and kept at room conditions with dry air. The weight of each leaf was measured at 0 and 22 hours, and then the leaves were dried to a constant weight. Water content in fresh leaves was 1.1 % greater in elevated O_3 than in ambient O_3, but the difference was non-significant (data not shown). Furthermore, at 22 hours, leaves obtained from elevated O_3 lost 6.9 % more water than those obtained from ambient O_3, however the difference was non-significant (data not shown). Therefore larval feeding was not affected by O_3-mediated alteration in the dehydration of leaves.

The average fresh weight of larvae, faeces, and consumed leaf, and the gained weight were determined by an analytical balance (Mettler® M22). To assess the effects on larval feeding and conversion of food to biomass, consumption and utilization of ozonated and

189 control tissues were compared by means of nutritional indices (Kogan 1986; Waldbaure
190 1968) for the 2^{nd}, 3^{rd}, 4^{th}, 5^{th}, 6^{th}, and 7^{th} instars using standard gravimetric procedures.
191 Consumption index (CI), mass growth rate (GR), ECD; ECI, and AD were calculated
192 using standard gravimetric procedures described by Waldbauer (1968):

193 a) CI=C/ [T×A], where C is the fresh weight of leaf consumed, T is the duration of
194 feeding period and A is the mean fresh weight of the larvae during the feeding
195 period. CI measures the amount of food eaten per unit time relative to mean
196 weight of larvae during the feeding period.

197 b) GR= G/[T×A], where G is the fresh weight gain of the larvae. GR measures the
198 amount of weight gained per unit time relative to the mean weight of the larvae
199 during the feeding period.

200 c) ECI= (G/C) x (100). ECI is an overall measure of the larvae's ability to utilize
201 ingested food for growth.

202 d) ECD= [G/(C-F)] x (100), where F is the weight of faeces during the feeding
203 period. ECD is an overall measure of the larvae ability to utilize digested food for
204 growth.

205 e) AD= [(C-F)/C] x (100). AD measures the larvae's ability to digest the introduced
206 food.

207 Exuviae were measured with the faeces since they are not a part of the insect at the end of
208 the experiment (Reese and Beck 1976; Abu ElEla and ElSayed 2015, Abu ElEla et al
209 2016).

210 **2.3. Data Analysis**

211 Four values were used per O_3 treatment per instar stage, each of which was a robust
212 estimate of one independent experimental unit. The alpha level was predefined at α=0.05.
213 The data of CI, GR, ECI, ECD and AD were not satisfactorily fit to Gaussian distribution
214 and therefore were subjected to a Box-Cox power transformation (Box and Cox 1964), as
215 described by Agathokleous et al. (2016b). Data of each response variable were subjected
216 to repeated measures Analysis of Variances (rANOVAs) where Instar was the
217 within-subjects factor with 6 levels and O_3 the categorical predictor with 2 levels.
218 rANOVA was based on *effective hypothesis* Sums of Squares (*Type 6 SS*) straightforward
219 computation method (Hocking 2013) with σ-restricted coding of effects (Hill and Lewicki
220 2006). Type 6 *SS* for each effect is the difference of the model *SS* for all the other effects
221 from the whole model *SS*, thus, providing an explicit estimate of predicted values
222 variability for the outcome that is attributed to each effect (Hill and Lewicki 2006). When
223 rANOVAs returned overall significances at a level of significance α=0.05 (H_0 rejected),
224 the data were further subjected to Bonferroni *a posteriori* test.

225 Data were processed and statistically analyzed in the software MS EXCEL 2010 (©
226 Microsoft) and STATISTICA v.10 (© StatSoft Inc.).
227
228 **2. RESULTS AND DISCUSSION**
229 Based on observations, larvae from ambient treatment needed 24 days from 1^{st} to 7^{th}
230 instar, whereas larvae from elevated O_3 needed 21 days from 1^{st} to 7^{th} instar.
231 **GR** (Fig 1*A*) significantly varied among instar stages (F=20.0 P<0.001). Second and 4^{th}
232 instars showed similar GR which was on average 4.3 times greater than that of 3^{rd}, 5^{th}, 6^{th}
233 and 7^{th} instars (P<0.05). Third, 5^{th} and 6^{th} instars shared a similar GR, which was on
234 average 3.5 times lower than that of 2^{nd} and 4^{th} instars and 4 times greater than that of 7^{th}
235 instar. These differences were significant except for GR between 6^{th} and 7^{th} instars due to
236 large relative standard deviation (RSD) in 6^{th} instar (P<0.05). Seventh instars had a
237 multi-fold lower GR than 2^{nd}, 3^{rd}, 4^{th}, and 5^{th} instars. Larvae displayed a 137 % greater
238 GR in elevated O_3 than in ambient O_3 (F=9.2, P<0.05). O_3 effects did not vary
239 significantly among instar stages (F=1.8, P=0.152).
240 **CI** (Fig 1*B*) also varied among instar stages (F=13.8, P<0.001). Second instars had on
241 average 2.6 times greater CI than 3^{rd}, 5^{th}, 6^{th} and 7^{th} instars (P<0.05); they also had 1.7
242 times greater CI than 4^{th} instars, however the difference was non-significant. Seventh

243 instars had significantly lower CI than 2nd, 3rd and 4th instars. Larvae showed a 115 %
244 greater CI in elevated O_3 than in ambient O_3 (F=9.0, P<0.05), while O_3 effects did not
245 vary significantly among instar stages (F=0.3, P=0.919). When food contains less
246 nitrogen, the consumption by insects increases to compensate for nitrogen acquisition.
247 Thus, elevated O_3-treated leaves, might have lower nitrogen content than elevated
248 O_3-treated leaves, however this could only be a speculation as we have no supportive data.
249 In the experiment of Agathokleous et al. (2017a), leaves of white birch trees were
250 collected in July, after a similar O_3 exposure. By that time, leaves exposed to O_3 had
251 lower content of total phenolics and condensed tannin than leaves, but no different leaf
252 mass per area (LMA), compared to leaves exposed to ambient O_3. In that no-choice
253 laboratory assay, 2nd instar larvae and adults of the leaf beetle did not significantly
254 increase the leaf consumption to compensate for degraded leaf palatability caused by O_3.
255 Several other investigations reported that insects showed preference towards O_3-treated
256 leaf material. For example, the monarch butterfly, *Danaus plexippus* (Lepidoptera:
257 Nymphalidae), preferred O_3-treated leaves of *Asclepias curassavica* and *A. syriaca*
258 (Bolsinger *et al.* 1992), and the Mexican bean beetle, *Epilachna varivestis* (Coleoptera:
259 Coccinellidae), preferred O_3-treated leaves of soybean, *Glycine max* (L.) Merr. (Endress
260 and Post 1985; Chappelka et al. 1988). Jones and Coleman (1988a) found that the willow

leaf beetle, *Plagiodera versicolora* (Coleoptera: Chrysomelidae), not only preferred O_3-treated plants but also consumed more foliage. This phenomenon might be upon decreased palatability and/or reduced defense of the leaves. Consumption of leaf area alone is not an efficient indicator of O_3-induced alterations because of the several factors which interplay. This assumption relies on the fact that consumption alone cannot inform if changes are upon O_3-induced changes in the palatability or defense of leaves, or if changes in consumption have any effects on insect physiological performance (Whittaker et al. 1989).

The extent to which leaf palatability is improved depends on the O_3 exposure and the subsequent plant response. Moderate increases in the levels of chemicals produced by plants may translate to stimulation of insect performance, i.e. hormesis (Ali and Agrawal 2012; Agathokleous 2018). The increase in CI, due to treatment of leaves with O_3, observed in this experiment is in agreement with the GR results, thus verifying earlier findings where the amount of growth reduction was generally proportional to reduced food consumption (Woodring et al. 1978; Adham et al. 2005a; Abu ElEla et al. 2016). The present results may hint to O_3-induced increase in the palatability and decrease in the defense of the leaves, something that requires further investigations.

278 **ECI** (Fig 2*A*) showed differences among instar stages (F=32.0, P<0.001). Independently
279 from O_3, 3rd and 4th instars had no significantly different ECI from 2nd instars, whereas 5th
280 and 6th instars had lower ECI compared to 2nd instars. Seventh instars had a multi-fold
281 lower ECI than all the previous instars. Instars showed a greater ECI in elevated O_3 than
282 in ambient O_3 (F=11.8, P<0.05), and O_3 effects did vary significantly among instar stages
283 (F=7.6, P<0.001). Within each instar stage, the only significant difference was observed
284 in the 3rd instars. Instars of ambient O_3 had much lower ECI at 3rd than at 2nd stage,
285 whereas instars of elevated O_3 had no significantly different ECI between 2nd and 3rd
286 stages. Thus, 3rd instars of elevated O_3 had greater ECI than 3rd instars of ambient O_3.
287 **ECD** (Fig 2*B*) also showed significant differences among instar stages (F=105.0,
288 P<0.001). Larvae of ambient or elevated O_3 showed lower ECD in 5th, 6th and 7th instar
289 stages than in 4th one. Seventh instars had a multi-fold lower ECD than all the previous
290 instars. Fourth instars larvae were more selective feeders and choose more digestive
291 foliage from the inter-vein regions of the leaf. Also, their metabolic rate was greater than
292 other instar stages, and, hence, more digested food was available for conversion to body
293 substance (i.e. ECD) (Abu ElEla and ElSayed 2015). It was noticed that 5th, 6th and 7th
294 instar larvae were more generalized in feeding, and they ingested different parts of
295 foliage such as leaf veins, which contain large quantities of indigestible crude fiber.

296 Therefore, it is likely that the 7th instar larvae had lower metabolic rate than younger ones
297 (2nd instars). The decrease in ECD indicates a less precise correspondence between larval
298 requirements and the level of nutrients balance in the diet. Larvae showed a greater ECD
299 in elevated O_3 than in ambient O_3 (F=43.5, P<0.001). ECD may decrease as a result of a
300 compensation to increase nutrient intake in leaves with reduced nutrients, along with
301 parallel intake of toxins. Our results are reverse, suggesting that there was no issue of
302 O_3-induced production of toxins in leaves. The O_3-induced increased efficiency of larvae
303 to convert ingested and digested food (ECI and ECD, Fig. 2*A,B*) observed in this study
304 can explain the enhanced GR (Fig 1*A*). O_3 effects did vary significantly among instar
305 stages (F=28.1, P<0.001). Within instars, the only difference was a lower ECD in larvae
306 of ambient than in larvae of elevated O_3 at 3rd and 7th stages. Larvae of elevated O_3
307 displayed a significantly greater ECD in 3rd and 4th instar than in 2nd instar, whereas
308 larvae of ambient O_3 displayed lower ECD in 3rd stage and statistically non-different
309 ECD in 4th stage compared to 2nd stage. Greatest ECD was recorded in 3rd and 4th instar
310 stages, when larvae were fed with elevated O_3-treated leaves, in agreement with findings
311 in *Helicoverpa armigera* Hübner (Lepidoptera: Noctuidae) (Hemati et al. 2012).
312 **AD** measures the digestive availability to the larvae, which is an important aspect in a
313 diet (Cohen 2005). AD (Fig 2*B*) displayed a variation within instars (F=104.9, P<0.001).

Seventh instars had greater AD than all the previous instars. Third, 4th and 5th instars had lower AD than 2nd instars. Sixth instar had significantly non-different AD than 2nd instar. Larvae displayed a lower AD in elevated O_3 than in ambient O_3 (F=47.1, P<0.001). Lower AD values may indicate lower suitability of leaf tissue for the larvae (Rahmathulla and Suresh 2012). However, the present results verify that the absorptive capacity of larvae (i.e. AD) is inversely proportional to ECD and ECI, as previously suggested (Waldbauer 1968; Xue et al. 2010; Teimouri et al. 2015). Increased consumption would accelerate passage of food through the gut and thereby reduce AD. In our research with the leaf beetle, we found that larvae reared on leaves treated with O_3 showed an increase in the consumption rates and thereby a decreased AD. Lower AD of the leaf beetle larvae indicates an adaptation to compensate for an increase in ECD and ECI which may result from a nutritional imbalance. For example, Adham et al. (2005a,b) showed that the 6th instar of *Spodoptera littoralis* (Lepidoptera: Noctuidae) larvae compensated for lower ECD by showing higher AD. O_3 effects did vary significantly among instar stages (F=41.0, P<0.001). Larvae of ambient O_3 had non-different AD in 3rd instar compared to 2nd instar but they had lower AD in 4th, 5th and 6th instars and greater AD in 7th instar than in 2nd instar; AD was similar among 4th, 5th and 6th instars. Larvae of elevated O_3 had

lower AD in 3rd, 4th and 5th instars and greater in 7th instar than in 2nd instar, but they had non-different AD between 6th and 2nd instars and among 3rd, 4th and 5th instars.

Larvae of elevated O_3 had lower AD in 3rd, 4th and 5th instar stages and greater AD in 6th stage than larvae of ambient O_3. Among all instar stages, larvae at 3rd instar were the most nutritionally responsive to O_3, as indicated by ECI, ECD and AD. In an earlier experiment, ECI, ECD and AD of 3rd and 4th instar larvae of the monarch butterfly (*Danaus plexippus* L.) fed with leaf tissues of *Asclepias curassavica* L. and *A. syriaca* L. were not different between O_3-treated and O_3-untreated tissues (Bolsinger et al., 1992). However, small leaf disks were used in that assay, and the indices were calculated only after a short exposure (maximum 24 h) of insects to the tissues. In our assay, larvae were provided with fresh leaves on a daily base and followed over their larval life cycle. AD may not be a sensitive index to changes in leaf secondary compounds as previously found in larvae of *A. alni* L. (Firidin and Mutlu 2009).

Rapid growth of larvae may be associated with increased gross feed efficiency (Medrano and Gall, 1975). Larvae may display a rapid growth (growth = CI × ECD × AD) as a result of a nutritional overcompensation if O_3-treated leaves have any costly effects (Manuwoto and Scriber 1985; Rahmathulla and Suresh 2012). In our assays, larvae fed with elevated O_3-treated leaved (M=287.7 ±50.7) had greater growth (F=25.9, P<0.01)

than larvae fed with ambient O_3-treated leaves (M=228.1 ±57.3), independently from larval stage. Therefore, larvae may have displayed a nutritional overcompensation. When insects consume leaf tissues which lack materials for the development of their body, ECI and ECD are expected to decrease whereas AD to increase (Teimouri et al. 2015). Our observations were reverse, suggesting that the leaf tissue quality was not degraded. When nutrients are less abundant in leaves, insects increase their consumption rate, accelerate food passage through their guts, and decrease AD. More research is required to address these effects across generations of insects fed with leaves grown under elevated O_3.

It should be noted that the results may differ at field because O_3 may transform the scent or degrade leaf-emitting volatile organic compounds (VOCs), becoming thus a repellant to herbivores and imbedding the plant-insect communication (Fuhrer and Booker 2003; Lindroth 2010; Fuentes et al. 2013; Blande et al. 2014; Cui et al. 2014; Farré-Armengol et al. 2016; Li et al. 2016). Nonetheless, the results of the present study are the first of their kind and are still valuable because in an O_3-polluted environment insects will not have the choice to select among O_3 conditions and thus will have to graze leaves at the relevant area.

3. CONCLUSIONS

- O_3 does indirectly change the growth and nutrition of the leaf beetle larvae.
- O_3 treatment of leaves may enhance the insect performance which may be proved costly for the plants under field conditions.
- ECD and AD can be utilized as efficient biomarkers of O_3 injury.
- Third instars can serve as the most effective O_3 bioindicator among all the larval instars of the leaf beetle.
- When needed, control of the leaf beetle at an O_3-enriched environment should be conducted before the 4^{th} instar stage where larvae can cause greater injuries to plants (greater ECI and ECD).
- Indirect O_3-induced alterations of insect physiology through consumption of ozonated leaf tissues require further experimentations to reveal potential consequences over insect generations.

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Fig 1. Means ± SE (n = 4) of growth rate (GR) (*A*) and consumption index (CI) (*B*) of 2nd, 3rd, 4th, 5th, 6th, and 7th larvae instars of the leaf beetle *Agelastica coerulea* (Baly, 1874) (hereafter leaf beetle) fed with leaves of Japanese white birch (*Betula platyphylla* var. *japonica*) obtained from ambient or elevated O_3 atmospheres, under laboratory conditions. Asterisks indicate rANOVA significant effects at $P<0.05$ (*), $P<0.01$ (**) and $P<0.001$ (***), whereas "n.s" indicates non-significant effects ($P>0.05$). Different lowercase letters above instar stages show statistically significant differences among the instar stages (O_3 pooled). Differences are marked according to Bonferroni test (α=0.05).

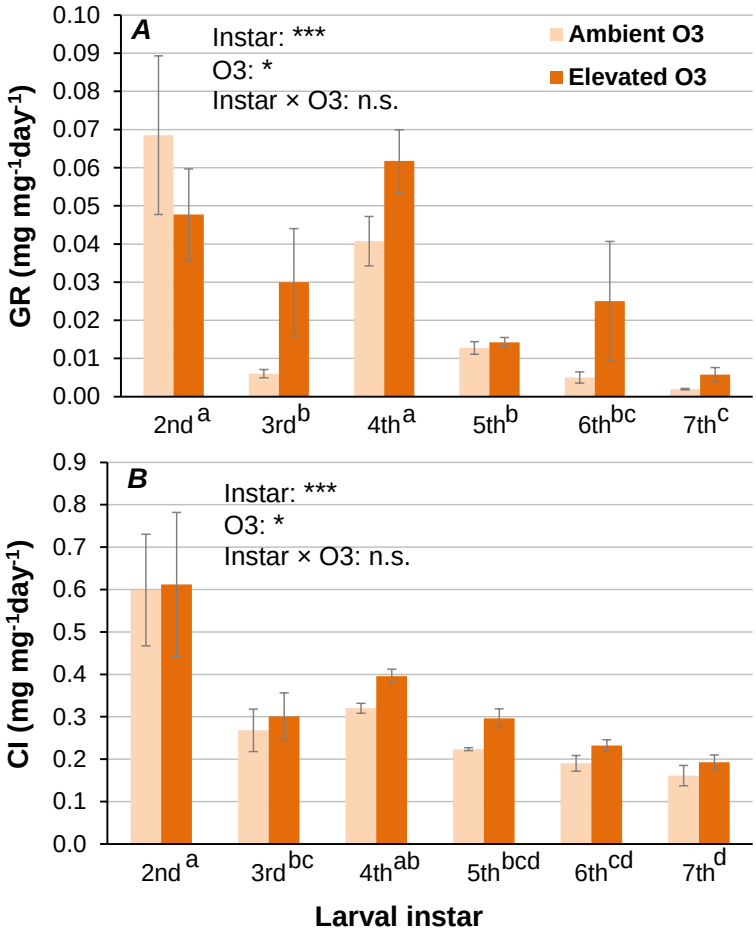

Fig 2. Means ± SE (n = 4) of efficiency conversion of ingested food (ECI) (*A*), efficiency conversion of digested food (ECD) (*B*), and approximate digestibility (AD) (*C*) of 2nd, 3rd, 4th, 5th, 6th, and 7th larvae instars of the leaf beetle *Agelastica coerulea* (Baly, 1874) (hereafter leaf beetle) fed with leaves of Japanese white birch (*Betula platyphylla* var. japonica) obtained from ambient or elevated O_3 atmospheres, under laboratory conditions. Asterisks indicate rANOVA significant effects at P<0.05 (*), P<0.01 (**) and P<0.001 (***), whereas "n.s" indicates non-significant effects (P>0.05). Different lowercase letters above instar stages show statistically significant differences among the instar stages (O_3 pooled). Different lowercase letters above the means show statistically significant differences within the interaction Instar × O_3. Differences are marked according to Bonferroni test (α=0.05).