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**Growth and nutrition of *Agelastica coerulea* (Coleoptera: Chrysomelidae) larvae
changed when fed with leaves obtained from an O₃-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 O₃-treated leaves of Japanese white birch (*Betula platyphylla* var. *japonica*). Larvae fed with O₃-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 O₃-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 O₃-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.*, O₃ 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 O₃-treated leaves in these assays. This study suggests insects may be more injurious to O₃-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 (O₃) 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 O₃ peaks in some regions of Europe (Vingarzan 2004; Derwent et al. 2007; Cape 2008; The Royal Society 2008; Sicard et al.

2016, 2017; Solomou et al. 2018). Northeast Asia is a hot spot of O₃ pollution (Nagashima et al. 2017; Trieu et al. 2017; Wang et al. 2017). Domestically-produced O₃ and transboundary transport from outside, mainly from China, result in significant increases in O₃ levels in Japan in recent years (Nagashima et al. 2010, 2017; Tanimoto 2009; Tanimoto et al. 2009). Furthermore, an increase in background O₃ levels in regions of the northern hemisphere may occur in the near future (The Royal Society 2008). O₃ is a gaseous pollutant which causes toxicity to plants at macro- and microscopic levels, when its concentrations exceed species-specific thresholds, through reactions in the leaves, with a subsequent production of highly oxidative intermediates (Robinson and Rowland 1996; Oksanen et al. 2013; Vaultier and Jolivet 2015). Such exceedances may result in reduction in plant vigor, suppression of yields and productivity, with further implications to ecological processes and trophic cascades (Feng et al. 2008; Lindroth 2010; Ainsworth et al. 2012; Koike et al. 2013; Blande et al. 2014; Agathokleous et al. 2016a; Chappelka and Grulke 2016).

There has been a notable progress in research on O₃ effects on vegetation and plant ecosystems over the last decades (Feng et al. 2008; Lindroth 2010; Ainsworth et al. 2012; Koike et al. 2013; Blande et al. 2014; Agathokleous et al. 2015a,b, 2016); however, the knowledge about O₃ 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 O₃ 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 O₃, the level of defense chemicals produced by plants, and thereby the insect performance, depend on the exposure level or on the O₃ dose uptake by plants, within the framework of hormesis (Agathokleous 2018). For instance, insects may display a

83 preference towards leaves which experience short exposure to elevated O₃ levels and
84 have increased palatability (Jones and Coleman 1988a; Bolsinger et al. 1991, 1992).
85 Recently, results from laboratory assays showed that O₃ altered the feeding behavior of
86 the leaf beetle *Agelastica coerulea* (Baly 1874) (hereafter leaf beetle) into leaves of
87 Japanese white birch (*Betula platyphylla* var. *japonica*) (Agathokleous et al. 2017a). In
88 these assays, it was found that overwintered adults preferred elevated O₃-treated leaves
89 than ambient O₃-treated ones and that the feeding behavior of 2nd instar larvae was not
90 changed when larvae could select between ambient O₃-treated and elevated O₃-treated
91 leaves (Agathokleous et al. 2017a). These laboratory observations are in agreement with
92 observations in assays with the common leaf weevil *Phyllobius pyri* L. (Coleoptera:
93 Curculionidae) (Freiwald et al. 2008) and oppose earlier field observations where the leaf
94 beetle deterred from grazing leaves of Japanese white birch in elevated O₃ sites of a Free
95 Air Controlled Exposure (FACE) system (Sakikawa et al. 2014, 2016; Vanderstock et al.
96 2016), a phenomenon which may be upon O₃-induced degradation of biogenic volatile
97 organic compounds (BVOCs) that repels insects (Fuentes et al. 2013; Dötterl et al. 2016;
98 Farré-Armengol et al. 2016; Li et al. 2016). However, in O₃-polluted regions, herbivore
99 insects have no privilege to choose between O₃ sites, and, thus, feed on plants under
100 stress. The implications of this feeding to the insect nutrition remain unknown.

In the present study, we explored the possibility that O₃ does affect insect nutrition indirectly, when insects consume leaves which underwent elevated O₃ exposure. In order to identify and quantify O₃ effects on insect nutrition, we conducted laboratory assays with a collection of larvae instars of the leaf beetle fed with Japanese white birch leaves obtained from sites with either background or elevated O₃ levels in a FACE system. We hypothesized that O₃ would have indirect effects on the nutrition of larvae feeding with O₃-treated leaves, and the effects could vary among larval instars which differ in their anatomical and physiological characteristics.

We assessed larvae consumption, mass growth, efficiency of conversion of ingested food (growth efficiency, ECI), efficiency of conversion of digested food (ECD), and assimilation efficiency (approximate digestibility, AD) as effective indicators of food utilization by insects (Slansky 1985). ECI relates the total consumption (food ingested) of insect to the amount of body mass, whereas ECD ignores undigested food (Slansky 1985; Farrar et al. 1989). AD indicates the food digestibility whereas ECI and ECD indicate the insect efficiency to convert food into body mass.

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°03.6'N 141°19.7'E (Japan Meteorological Agency 2017). For the period May-August, the main meteorological conditions (mean $\pm$ s.e.) were: mean monthly average of air temperature = 18.95 ($\pm$ 2.06) °C; daily maximum temperature = 23.48 ($\pm$ 1.95) °C; daily minimum temperature = 15.50 ($\pm$ 2.18) °C; wind speed = 4.00 ($\pm$ 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 O₃-FACE system consisted of six rings; three with ambient air and three with ambient air enriched with O₃ (target = 70 nmol mol⁻¹) during daylight hours with photosynthetic photon flux density (PPFD) > 70 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (07:00–17:00). In 2014, the plants were exposed to elevated O₃ from August 15 to October 26, and, in 2015, from April 24 to October 26. The mean O₃ levels (07:00–17:00) for elevated O₃ plots were 60 nmol mol⁻¹ in 2014 and 72 nmol mol⁻¹ in 2015, whereas for ambient O₃ plots were 20 nmol mol⁻¹ in 2014 and 34 nmol mol⁻¹ in 2015. In 2016, the O₃ enrichment started on May 18, and the last leaf samples were collected on July 15. The mean O₃ levels (07:00–17:00) in elevated O₃ 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 O₃ levels were recorded from June 1 onwards. Ambient O₃ 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 O₃ exposures can be found in Agathokleous et al. (2017b).

2.2. Laboratory insect culture and assays

The colony of the leaf beetle was started with egg patches obtained from ambient air conditions of Sapporo Experimental Forest of Hokkaido University, on June 16, 2016. Deposited eggs were selected based on the intensity of yellow color (eggs close to hatching avoided) and transferred to laboratory. Thereafter, all the processes were conducted under laboratory conditions. Eggs hatching occurred within four to five days from the collection. Newly hatched larvae were placed in plastic boxes (12 × 6 × 18 cm). Ten larvae were placed in a box replicated four times per O₃ treatment per instar stage, giving thus a total of 40 individual larvae per instar per O₃ treatment. The assays were conducted based on a completely randomized design, and the position of the boxes was rotated on a daily base. The initial weight of the larvae was measured and the culture was kept at a temperature of 25 ± 2°C, 65 ± 5 % relative humidity and 14:10 hours (D: L) photoperiod (Abu ElEla and ElSayed 2015; Adham et al 2005 a,b; Abu ElEla et al 2016). Larvae of each preceding instar molted and transferred to next stage by shedding the skin. Larvae were fed on weighed quantities of treated and untreated fresh mature leaves of Japanese white birch leaves until pupation. Fresh late leaves were collected from ambient and elevated O₃ plots and provided to the larvae on a daily base, until the end of larval 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 O₃ 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 O₃ than in ambient O₃, but the difference was non-significant (data not shown). Furthermore, at 22 hours, leaves obtained from elevated O₃ lost 6.9 % more water than those obtained from ambient O₃, however the difference was non-significant (data not shown). Therefore larval feeding was not affected by O₃-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

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

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

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

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

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

e) $AD = [(C-F)/C] \times (100)$. AD measures the larvae's ability to digest the introduced food.

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

2.3. Data Analysis

Four values were used per O₃ treatment per instar stage, each of which was a robust estimate of one independent experimental unit. The alpha level was predefined at $\alpha=0.05$. The data of CI, GR, ECI, ECD and AD were not satisfactorily fit to Gaussian distribution and therefore were subjected to a Box-Cox power transformation (Box and Cox 1964), as described by Agathokleous et al. (2016b). Data of each response variable were subjected to repeated measures Analysis of Variances (rANOVAs) where Instar was the within-subjects factor with 6 levels and O₃ the categorical predictor with 2 levels. rANOVA was based on *effective hypothesis* Sums of Squares (*Type 6 SS*) straightforward computation method (Hocking 2013) with σ -restricted coding of effects (Hill and Lewicki 2006). Type 6 SS for each effect is the difference of the model SS for all the other effects from the whole model SS, thus, providing an explicit estimate of predicted values variability for the outcome that is attributed to each effect (Hill and Lewicki 2006). When rANOVAs returned overall significances at a level of significance $\alpha=0.05$ (H₀ rejected), the data were further subjected to Bonferroni *a posteriori* test.

Data were processed and statistically analyzed in the software MS EXCEL 2010 (© Microsoft) and STATISTICA v.10 (© StatSoft Inc.).

2. RESULTS AND DISCUSSION

Based on observations, larvae from ambient treatment needed 24 days from 1st to 7th instar, whereas larvae from elevated O₃ needed 21 days from 1st to 7th instar.

GR (Fig 1A) significantly varied among instar stages ($F=20.0$ $P<0.001$). Second and 4th instars showed similar GR which was on average 4.3 times greater than that of 3rd, 5th, 6th and 7th instars ($P<0.05$). Third, 5th and 6th instars shared a similar GR, which was on average 3.5 times lower than that of 2nd and 4th instars and 4 times greater than that of 7th instar. These differences were significant except for GR between 6th and 7th instars due to large relative standard deviation (RSD) in 6th instar ($P<0.05$). Seventh instars had a multi-fold lower GR than 2nd, 3rd, 4th, and 5th instars. Larvae displayed a 137 % greater GR in elevated O₃ than in ambient O₃ ($F=9.2$, $P<0.05$). O₃ effects did not vary significantly among instar stages ($F=1.8$, $P=0.152$).

CI (Fig 1B) also varied among instar stages ($F=13.8$, $P<0.001$). Second instars had on average 2.6 times greater CI than 3rd, 5th, 6th and 7th instars ($P<0.05$); they also had 1.7 times greater CI than 4th instars, however the difference was non-significant. Seventh

instars had significantly lower CI than 2nd, 3rd and 4th instars. Larvae showed a 115 % greater CI in elevated O₃ than in ambient O₃ ($F=9.0$, $P<0.05$), while O₃ effects did not vary significantly among instar stages ($F=0.3$, $P=0.919$). When food contains less nitrogen, the consumption by insects increases to compensate for nitrogen acquisition. Thus, elevated O₃-treated leaves, might have lower nitrogen content than elevated O₃-treated leaves, however this could only be a speculation as we have no supportive data. In the experiment of Agathokleous et al. (2017a), leaves of white birch trees were collected in July, after a similar O₃ exposure. By that time, leaves exposed to O₃ had lower content of total phenolics and condensed tannin than leaves, but no different leaf mass per area (LMA), compared to leaves exposed to ambient O₃. In that no-choice laboratory assay, 2nd instar larvae and adults of the leaf beetle did not significantly increase the leaf consumption to compensate for degraded leaf palatability caused by O₃. Several other investigations reported that insects showed preference towards O₃-treated leaf material. For example, the monarch butterfly, *Danaus plexippus* (Lepidoptera: Nymphalidae), preferred O₃-treated leaves of *Asclepias curassavica* and *A. syriaca* (Bolsinger et al. 1992), and the Mexican bean beetle, *Epilachna varivestis* (Coleoptera: Coccinellidae), preferred O₃-treated leaves of soybean, *Glycine max* (L.) Merr. (Endress and Post 1985; Chappelka et al. 1988). Jones and Coleman (1988a) found that the willow

leaf beetle, *Plagioder a versicolor a* (Coleoptera: Chrysomelidae), not only preferred O₃-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 O₃-induced alterations because of the several factors which interplay. This assumption relies on the fact that consumption alone cannot inform if changes are upon O₃-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 O₃ 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 O₃, 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 O₃-induced increase in the palatability and decrease in the defense of the leaves, something that requires further investigations.

278 **ECI** (Fig 2A) 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 2B) 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.

Therefore, it is likely that the 7th instar larvae had lower metabolic rate than younger ones (2nd instars). The decrease in ECD indicates a less precise correspondence between larval requirements and the level of nutrients balance in the diet. Larvae showed a greater ECD in elevated O₃ than in ambient O₃ ($F=43.5$, $P<0.001$). ECD may decrease as a result of a compensation to increase nutrient intake in leaves with reduced nutrients, along with parallel intake of toxins. Our results are reverse, suggesting that there was no issue of O₃-induced production of toxins in leaves. The O₃-induced increased efficiency of larvae to convert ingested and digested food (ECI and ECD, Fig. 2A,B) observed in this study can explain the enhanced GR (Fig 1A). O₃ effects did vary significantly among instar stages ($F=28.1$, $P<0.001$). Within instars, the only difference was a lower ECD in larvae of ambient than in larvae of elevated O₃ at 3rd and 7th stages. Larvae of elevated O₃ displayed a significantly greater ECD in 3rd and 4th instar than in 2nd instar, whereas larvae of ambient O₃ displayed lower ECD in 3rd stage and statistically non-different ECD in 4th stage compared to 2nd stage. Greatest ECD was recorded in 3rd and 4th instar stages, when larvae were fed with elevated O₃-treated leaves, in agreement with findings in *Helicoverpa armigera* Hübner (Lepidoptera: Noctuidae) (Hemati et al. 2012).

AD measures the digestive availability to the larvae, which is an important aspect in a diet (Cohen 2005). AD (Fig 2B) displayed a variation within instars ($F=104.9$, $P<0.001$).

314 Seventh instars had greater AD than all the previous instars. Third, 4th and 5th instars had
315 lower AD than 2nd instars. Sixth instar had significantly non-different AD than 2nd instar.
316 Larvae displayed a lower AD in elevated O₃ than in ambient O₃ ($F=47.1$, $P<0.001$).
317 Lower AD values may indicate lower suitability of leaf tissue for the larvae (Rahmathulla
318 and Suresh 2012). However, the present results verify that the absorptive capacity of
319 larvae (i.e. AD) is inversely proportional to ECD and ECI, as previously suggested
320 (Waldbauer 1968; Xue et al. 2010; Teimouri et al. 2015). Increased consumption would
321 accelerate passage of food through the gut and thereby reduce AD. In our research with
322 the leaf beetle, we found that larvae reared on leaves treated with O₃ showed an increase
323 in the consumption rates and thereby a decreased AD. Lower AD of the leaf beetle larvae
324 indicates an adaptation to compensate for an increase in ECD and ECI which may result
325 from a nutritional imbalance. For example, Adham et al. (2005a,b) showed that the 6th
326 instar of *Spodoptera littoralis* (Lepidoptera: Noctuidae) larvae compensated for lower
327 ECD by showing higher AD. O₃ effects did vary significantly among instar stages
328 ($F=41.0$, $P<0.001$). Larvae of ambient O₃ had non-different AD in 3rd instar compared to
329 2nd instar but they had lower AD in 4th, 5th and 6th instars and greater AD in 7th instar than
330 in 2nd instar; AD was similar among 4th, 5th and 6th instars. Larvae of elevated O₃ 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 O₃ had lower AD in 3rd, 4th and 5th instar stages and greater AD in 6th stage than larvae of ambient O₃. Among all instar stages, larvae at 3rd instar were the most nutritionally responsive to O₃, 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 O₃-treated and O₃-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 ($\text{growth} = \text{CI} \times \text{ECD} \times \text{AD}$) as a result of a nutritional overcompensation if O₃-treated leaves have any costly effects (Manuwoto and Scriber 1985; Rahmathulla and Suresh 2012). In our assays, larvae fed with elevated O₃-treated leaved (M=287.7 ±50.7) had greater growth ($F=25.9$, $P<0.01$)

than larvae fed with ambient O₃-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 O₃.

It should be noted that the results may differ at field because O₃ may transform the scent or degrade leaf-emitting volatile organic compounds (VOCs), becoming thus a repellent 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 O₃-polluted environment insects will not have the choice to select among O₃ conditions and thus will have to graze leaves at the relevant area.

3. CONCLUSIONS

- O₃ does indirectly change the growth and nutrition of the leaf beetle larvae.
- O₃ 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 O₃ injury.
- Third instars can serve as the most effective O₃ bioindicator among all the larval instars of the leaf beetle.
- When needed, control of the leaf beetle at an O₃-enriched environment should be conducted before the 4th instar stage where larvae can cause greater injuries to plants (greater ECI and ECD).
- Indirect O₃-induced alterations of insect physiology through consumption of ozonated leaf tissues require further experimentations to reveal potential consequences over insect generations.

References

- Abu ElEla SA, ElSayed WM (2015) The influence of cadmium on the food consumption and utilization of the cotton leaf worm *Spodoptera littoralis* (Boisd.) (Lepidoptera: Noctuidae). Ecol Balkan 7:81–85.
- Abu ElEla SA, Nassar MM, Eesa NN (2016) Impact of lead acetate on quantitative

384 consumption and utilization of the cotton leaf worm, *Spodoptera littoralis*
385 (Boisduval, 1833) (Lepidoptera: Noctuidae). Ecol Balkan 8: 101–106.

386 Adham FK, Gabre RM, Abu El-Ela SA, Hassan MM (2005a) Growth and feeding
387 efficiency of cotton leaf worm *Spodoptera littoralis* (Boisd.) (Lepidoptera:
388 Noctuidae) on cotton plant *Gossypium barbadens* (Malvaceae) grown in
389 enriched CO₂ atmosphere. Bul Entom Soc Egy 82:187–196.

390 Adham FK, Gabre RM, Abu El-Ela SA (2005b) The performance parameters of
391 *Spodoptera littoralis* (Boisd.) (Lepidoptera: Noctuidae) fed on cotton leaves
392 grown in enriched CO₂ atmosphere. Bull Entom Soc Egy 82:197–205.

393 Agathokleous E (2018) Environmental hormesis, a fundamental non-monotonic
394 biological phenomenon with implications in ecotoxicology and environmental
395 safety. Ecotox Environ Safe 148:1042–1053. DOI:
396 10.1016/j.ecoenv.2017.12.003 [In Press]

397 Agathokleous E, Koike T, Watanabe M, Hoshika Y, Saitanis CJ (2015a) Ethylene-di-urea
398 (EDU), an effective phytoprotectant against O₃ deleterious effects and a valuable
399 research tool. J Agric Meteor 71:185–195.

400 Agathokleous E, Saitanis CJ, Koike T (2015b) Tropospheric O₃, the nightmare of wild
401 plants: A review study. J Agric Meteor 71:142–152.

402 Agathokleous E, Saitanis CJ, Wang X, Watanabe M, Koike T (2016a) A review study on
 403 past 40 years of research on effects of tropospheric O₃ on belowground structure,
 404 functioning and processes of trees: a linkage with potential ecological
 405 implications. *Wat Air Soil Poll* 227:33.

406 Agathokleous E, Saitanis CJ, Stamatelopoulos D, Mouzaki-Paxinou A-C, Paoletti E,
 407 Manning WJ (2016b) Olive oil for dressing plant leaves so as to avoid O₃ injury.
 408 *Wat Air Soil Poll* 227:282.

409 Agathokleous E, Sakikawa T, Abu ElEla SA, Mochizuki T, Nakamura M, Watanabe M,
 410 Kawamura K, Koike T (2017a) Ozone alters the feeding behavior of the leaf
 411 beetle *Agelastica coerulea* (Coleoptera: Chrysomelidae) into leaves of Japanese
 412 white birch (*Betula platyphylla* var. *japonica*). *Environ Sci Poll Res* 24:
 413 17577–17583.

414 Agathokleous E, Vanderstock A, Kita K, Koike T (2017b) Stem and crown growth of
 415 Japanese larch and its hybrid F₁ grown in two soils and exposed to two free-air
 416 O₃ regimes. *Environ Sci Poll Res* 24:6634–6647.

417 Ainsworth EA, Yendrek CR, Sitch S, Collins WJ, Emberson LD (2012) The effects of
 418 tropospheric ozone on net primary productivity and implications for climate
 419 change. *Annu Rev Plant Biol* 63:637–661.

420 Ali JG, Agrawal AA (2012) Specialist versus generalist insect herbivores and plant
 421 defense. *Trends Plant Sci* 17:293–302.

422 Alstad DN, Edmunds GFJr, Weinstein LH (1982) Effects of air pollutants on insect
 423 populations. *Ann Rev Entomol* 27:369–384.

424 Blande JD, Holopainen JK, Niinements Ü (2014) Plant volatiles in polluted atmospheres:
 425 stress responses and signal degradation. *Plant Cell Environ* 37:1892–1904.

426 Bolsinger M, Lier ME, Lansky DM, Hughes PR (1991) Influence of ozone air pollution
 427 on plant-herbivore interactions. Part 1: biochemical changes in ornamental
 428 milkweed (*Asclepias curassavica* L.; Asclepiadaceae) induced by ozone. *Environ*
 429 *Pollut* 72:69–83.

430 Bolsinger M, Lier ME, Hughes PR (1992) Influence of ozone air pollution on
 431 plant-herbivore interactions. Part 2: Effects of ozone on feeding preference,
 432 growth and consumption rates of monarch butterflies (*Danaus plexippus*).
 433 *Environ Pollut* 77:31–37.

434 Box GEP, Cox DR (1964) An analysis of transformations. *J Roy Stat Soc B* 26:211–252.

435 Cape JN (2008) Surface ozone concentrations and ecosystem health: Past trends and a
 436 guide to future projections. *Sci Tot Environ* 400:257–269.

437 Chappelka A H, Grulke NE (2016) Disruption of the ‘disease triangle’ by chemical and

438 physical environmental change. Plant Biol 18:5–12.

439 Chappelka AH, Kraemer ME, Mebrahtu T, Rangappa M, Benepal PS (1988) Effects of
 440 ozone on soybean resistance to the Mexican bean beetle (*Epilachna varivestis*
 441 mulsant). Environ Exp Bot 28:53–66.

442 Cohen A (2015) Insect diets: science and technology, 2nd edition. CRC Press, Florida. 473
 443 pp.

444 Cui H, Su J, Wei J, Hu Y, Ge F (2014) Elevated O₃ enhances the attraction of
 445 whitefly-infested tomato plants to *Encarsia formosa*. Sci Rep 4:5350.

446 Derwent RG, Simmonds PG, Manning AJ, Spain TG (2007) Trends over a 20-year period
 447 from 1987 to 2007 in surface ozone at the atmospheric research station, Mace
 448 Head, Ireland. Atmos Environ 41:9091–9098.

449 Dötterl S, Vater M, Rupp T, Held A (2016) Ozone differentially affects perception of
 450 plant volatiles in western honey bees. J Chem Ecol 42:486–489.

451 Endress AG, Post SL (1985) Altered feeding preference of Mexican bean beetle
 452 *Epilachna varivestris* for ozonated soybean foliage. Environ Pollut 39:9–16.

453 Farrar RR, Barbour JD, Kennedy GG (1989) Quantifying food consumption and growth
 454 in insects. Ann Entomol Soc Am 82:593–598.

455 Farré-Armengol G, Peñuelas J, Li T, Yli-Pirilä P, Filella I, Llusia J, Blande JD (2016)

456 Ozone degrades floral scent and reduces pollinator attraction to flowers. New
 457 Phytol 209:152–160.

458 Feng Z, Kobayashi K, Ainsworth E (2008) Impact of elevated ozone concentration on
 459 growth, physiology, and yield of wheat (*Triticum aestivum* L.): a meta-analysis.
 460 Global Chang Biol 14:2696–2708.

461 Firidin B, Mutlu C (2009) Nitrogen utilization pattern and degradation capability of some
 462 plant secondary metabolites by *Agelastica alni* L. (Coleoptera: Chrysomelidae).
 463 J Entomol Res Soc 11:2.

464 Fred PH (1987) Interactions of insects, trees and air pollutants. Tree Physiol 3:93–102.

465 Freiwald V, Häikiö E, Julkunen-Tiitto R, Holopainen JK, Oksanen E (2008) Elevated
 466 ozone modifies the feeding behaviour of the common leaf weevil on hybrid
 467 aspen through shifts in developmental, chemical, and structural properties of
 468 leaves. Entomol Exp Applic 128:66–72.

469 Fuentes JD, Roulston TH, Zenker J (2013) Ozone impedes the ability of a herbivore to
 470 find its host. Environ Res Let 8:014048.

471 Fuhrer J, Booker F. (2003) Ecological issues related to ozone: agricultural issues. Environ
 472 Int 29:141–154.

473 Fuhrer J, Val Martin M, Mills G, Heald CL, Harmens H, Hayes F, Sharps K, Bender J,

474 Ashmore MR (2016). Current and future ozone risks to global terrestrial
 475 biodiversity and ecosystem processes. *Ecol Evol* 6:8785–8799.
 476 Hain FP (1987) Interactions of insects, trees and air pollutants. *Tree Physiol* 3:93–102.
 477 Hedges LV, Olkin I (1985) *Statistical methods for meta-analysis*, 1st edn. Academic Press,
 478 Orlando, FL. 369pp.
 479 Hemati SA, Naseri B, Ganbalani GN, Dastjerdi HR, Golizadeh A (2012). Effect of
 480 different host plants on nutritional indices of the pod borer, *Helicoverpa*
 481 *armigera*. *J Insect Sci* 12:55
 482 Hill T, Lewicki P (2006) *Statistics : methods and applications : a comprehensive reference*
 483 *for science, industry, and data mining*. StatSoft.
 484 Hillstrom ML, Lindroth RL (2008) Elevated atmospheric carbon dioxide and ozone alter
 485 forest insect abundance and community composition. *Insect Cons Diver*
 486 1:233–241.
 487 Hocking RR (1996) *Methods and Applications of Linear Models: Regression and the*
 488 *Analysis of Variance*, 3rd edition. New York, Wiley, 720p.
 489 Hughes PR (1988) Insect populations on host plants subjected to air pollution. In:
 490 Heinrichs EA (Ed), *Plant Stress-Insect Interactions*. Chichester, John Wiley, pp.
 491 249-319.

492 Japan Meteorological Agency (2017) <http://www.jma.go.jp/jma/indexe.html> Website
 493 accessed on 27th May 2017

494 Jones CG, Coleman JS (1988a) Plant stress and insect behavior: cottonwood, ozone and
 495 the feeding and oviposition preference of a beetle. *Oecologia* 76:51–56.

496 Jones CG, Coleman JS (1988b) Leaf disc size and insect feeding preference: implications
 497 for assays and studies on induction of plant defense. *Entom. Exp. Appl.*
 498 47:167–172.

499 Kogan M (1986) Bioassays for measuring quality of insect food. In: Miller JR, Miller TA
 500 (Eds.), *Insect-Plant Interactions.*, New York, Springer-Verlag, pp. 155-189.

501 Koike T (1995) Vegetation Science in Forestry: Global Perspective based on Forest
 502 Ecosystems of East & Southeast Asia. In: Box E.O. et al. (Eds.), *Physiological*
 503 *ecology of the growth characteristics of Japanese mountain birch in northern*
 504 *Japan: a comparison with Japanese mountain white birch*, Kluwer Academic
 505 Publishers, The Netherlands, pp.409–422.

506 Koike T, Tobita H, Shibata T, Mastuki S, Konno K, Kitao M, Yamashita N, Maruyama, Y.
 507 (2006) Defense characteristics of seral deciduous broad-leaved tree seedlings
 508 grown under differing levels of CO₂ and nitrogen. *Popul Ecol* 48:23–29.

509 Koike T, Watanabe M, Hoshika Y, Kitao M, Matsumura H, Funada R, Izuta T (2013)

510 Effects of ozone on forest ecosystems in East and Southeast Asia. In: Matyssek
 511 R, Clarke N, Cudlin P, Mikkelsen TN, Tuovinen J-P, Wieser G, Paoletti E (Eds.),
 512 Climate Change, Air Pollution and Global Challenges. Elsevier Pub. pp.
 513 371–390.

514 Lacey LA (1997) Manual of techniques in insect pathology. Bath, Academic Press, 409p.

515 Li T, Blande JD, Holopainen JK (2016) Atmospheric transformation of plant volatiles
 516 disrupts host plant finding. *Sci Rep* 6:33851.

517 Lindroth RL (2010) Impacts of elevated atmospheric CO₂ and O₃ on forests:
 518 phytochemistry, trophic interactions, and ecosystem dynamics. *J Chem Ecol*
 519 36:2–21.

520 Manuwoto S, Scriber JM (1985) Consumption and utilization of experimentally altered
 521 corn by southern armyworm: Iron, nitrogen, and cyclic hydroxamates. *J Chem*
 522 *Ecol* 11:1469–1483.

523 Matsuki S, Sano Y, Koike T (2004) Chemical and physical defense in the early and late
 524 leaves in three heterophyllous birch species native to northern Japan. *Annals Bot*
 525 93:141–147.

526 Mattson WJ, Haack RA (1987) Role of drought in outbreaks of plant-eating insects.
 527 *Bioscience* 37:110–118.

528 Medrano JF, Gall GAE (1975) Food consumption, feed efficiency, metabolic rate and
 529 utilization of glucose in lines of *Tribolium castaneum* selected for 21-day pupa
 530 weight. Genetics 83:393–407.

531 Nagashima T, Ohara T, Sudo K, Akimoto H (2010) The relative importance of various
 532 source regions on East Asian surface ozone. Atmos Chem Phys 10:11305–11322,

533 Nagashima T, Sudo K, Akimoto H, Kurokawa, J, Ohara T (2017) Long-term change in
 534 the source contribution to surface ozone over Japan. Atmos Chem Phys
 535 17:8231–8246.

536 Oksanen E, Pandey V, Keski-Saari S, Kontunen-Soppela S, Sharma C (2013) Impacts of
 537 increasing ozone on Indian plants. Environ Pollut 177:189–200.

538 Rahmathulla VK, Suresh HM (2012) Seasonal variation in food consumption,
 539 assimilation, and conversion efficiency of Indian bivoltine hybrid silkworm,
 540 *Bombyx mori*. J Insect Sci 12:82.

541 Reese JC, Beck SD (1976) Effects of allelochemics on the black cut worm, *Agrotis*
 542 *ipsilon*; effects of pbenzoquinone, hydroquinone, and duroquinone on larval
 543 growth, development, and utilization of food. Ann Entomol Soc Am 69:59–67.

544 Robinson JM, Rowland RA (1996) Carbohydrate and carbon metabolite accumulation
 545 responses in leaves of ozone tolerant and ozone susceptible spinach plants after

546 acute ozone exposure. *Photos Res* 50:103–115.

547 Royal Society (2008) Ground-level Ozone in the 21st Century: 1 Future Trends, Impacts
548 and Policy Implications. Science Policy Report 15/08.

549 Sakikawa T, Oikawa M, Watanabe M, Mao Q, Koike T (2014) The effect of ozone on leaf
550 phenology of white birch (*Betula platyphylla* var. *japonica*) grown under free-air
551 ozone exposure. *Boreal For Res* 62:59–60. (in Japanese)

552 Sakikawa T, Nakamura M, Watanabe M, Oikawa M, Satoh F, Koike T (2016) Leaf
553 phenology and insect grazing of Japanese white birch saplings grown under
554 free-air ozone exposure. *J Agr Meteorol* 72: 80–84.

555 Sas Institute. 2002. SAS/STAT User’s Guide. Version 9.1. SAS Institute, Cary, NC.

556 Sicard P, Serra R, Rossello P (2016) Spatiotemporal trends in ground-level ozone
557 concentrations and metrics in France over the time period 1999–2012. *Environ*
558 *Res* 149:122–144.

559 Sicard P, Anav A, De Marco A, Paoletti E (2017) Projected global tropospheric ozone
560 impacts on vegetation under different emission and climate scenarios. *Atmos*
561 *Chem Phys Discuss*. doi:10.5194/acp-2017-74

562 Slansky JrF (1985) Food utilization by insects: Interpretation of observed differences
563 between dry weight and energy efficiencies. *Entom Exp Applic* 39:47–60.

- 564 Smith CM, Khan ZR, Dutta Pathak M (1994) Techniques for Evaluating Insect
565 Resistance in Crop Plants. CRC Press LLC, 336p.
- 566 Solomou E, Poupkou A, Bolis S, Zanis P, Lazaridis M, Melas D (2018) Evaluating
567 near-surface ozone levels simulated from MACC global and regional modeling
568 systems in Eastern Mediterranean under the influence of Etesian winds. Atmos
569 Res. doi: 10.1016/j.atmosres.2017.09.010
- 570 Tanimoto H (2009) Increase in springtime tropospheric ozone at a mountainous site in
571 Japan for the period 1998–2006. Atmos Environ 43:1358–1363.
- 572 Tanimoto H, Ohara T, Uno I (2009) Asian anthropogenic emissions and decadal trends in
573 springtime tropospheric ozone over Japan: 1998–2007. Geophys Res Lett 36:
574 L23802.
- 575 Teimouri N, Sendi JJ, Zibae A, Khosravi R (2015) Feeding indices and enzymatic
576 activities of carob moth *Ectomyelois ceratoniae* (Zeller) (Lepidoptera:
577 Pyralidae) on two commercial pistachio cultivars and an artificial diet. J Saudi
578 Soc Agric Sci 14:76–82.
- 579 Trieu TT, Goto D, Yashiro H, Murata R, Sudo K, Tomita H, Satoh M, Nakajima T (2017)
580 Evaluation of summertime surface ozone in Kanto area of Japan using a
581 semi-regional model and observation. Atmos Environ 153:163–181.

582 Vaultier M-N, Jolivet Y (2015) Ozone sensing and early signaling in plants: An outline
 583 from the cloud. *Environ Exp Bot* 114:144–152.

584 Valkama E, Koricheva J, Oksanen E (2007) Effects of elevated O₃, alone and in
 585 combination with elevated CO₂, on tree leaf chemistry and insect herbivore
 586 performance: a meta-analysis. *Glob Change Biol* 13:184–201.

587 Vanderstock A, Agathokleous E, Inoue W, Eguchi N, Nakamura M, Satoh F, Kanie S,
 588 Koike T (2016) Preliminary survey on insect grazing in white birch stands under
 589 free-air O₃ fumigation. *Bor For Res* 64:27–29.

590 Vingarzan R (2004) A review of surface ozone background levels and trends. *Atmos*
 591 *Environ* 38:3431–3442.

592 Waldbauer GP (1968) The consumption and utilization of food by insects. *Advanc Ins*
 593 *Physiol* 5:229–288.

594 Wang T, Xue L, Brimblecombe P, Lam YF, Li L, Zhang L (2017) Ozone pollution in
 595 China: A review of concentrations, meteorological influences, chemical
 596 precursors, and effects. *Sci Tot Environ* 575:1582–1596.

597 White TCR (1974) A hypothesis to explain outbreaks of looper caterpillars, with special
 598 reference to populations of *Selidosema suavis* in a plantation of *Pinus radiata* in
 599 New Zealand. *Oecologia* 16:279–301.

600 White TCR (1976) Weather, food and plagues of locusts. *Oecologia* 22:119–134.

601 White TCR (1984) The abundance of invertebrate herbivores in relation to the availability
 602 of nitrogen in stressed plants. *Oecologia* 63:9C~105.

603 Whittaker JB, Kristiansen LW, Mikkelsen TN Moore R (1989) Responses to ozone of
 604 insects feeding on a crop and a weed species. *Environ Pollut* 62:89–101.

605 Wolf FM (1986) *Meta-analysis: Quantitative Methods for Research Synthesis*. Beverly
 606 Hills, CA: Sage.

607 Woodring JP, Clifford CW, Roe RM, Beckman BR (1978) Effects of CO₂ and anoxia on
 608 feeding, growth, metabolism, water balance, and blood composition in larval
 609 house crickets, *Acheta domesticus*. *J Ins Physiol* 24:499–509.

610 Xue M, Pang Y-H, Wang HT, Li Q-L, Liu TX (2010) Effects of four host plants on
 611 biology and food utilization of the cutworm, *Spodoptera litura*. *J Insect Sci*
 612 10:22.

613 Yli-Pirilä P, Copolovici L, Kännaste A, Noe S, Blande JD, Mikkonen S, Klemola T,
 614 Pulkkinen J, Virtanen A, Laaksonen A, Joutsensaari J, Niinemets Ü, Holopainen
 615 JK (2016) Herbivory by an outbreaking moth increases emissions of biogenic
 616 volatiles and leads to enhanced secondary organic aerosol formation capacity.
 617 *Environ Sci Tech* 50:11501–11510.

Fig 1. Means $\pm$ 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 O₃ 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 (O₃ pooled). Differences are marked according to Bonferroni test ($\alpha = 0.05$).

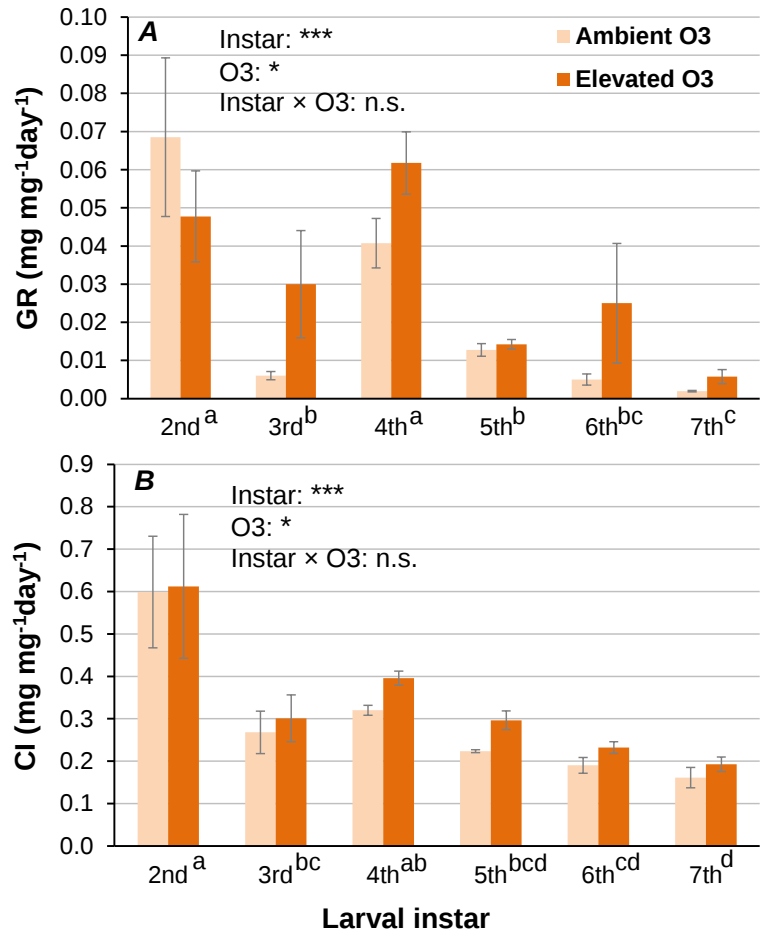


Fig 2. Means $\pm$ SE ($n = 4$) of efficiency conversion of ingested food (ECI) (A), efficiency conversion of digested food (ECD) (B), and approximate digestibility (AD) (C) of 2nd, 3rd, 4th, 5th, 6th, and 7th larvae instars of the leaf beetle *Agelastica coerulea* (Baly, 1874) (hereafter leaf beetle) fed with leaves of Japanese white birch (*Betula platyphylla* var. *japonica*) obtained from ambient or elevated O₃ 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 (O₃ pooled). Different lowercase letters above the means show statistically significant differences within the interaction Instar $\times$ O₃. Differences are marked according to Bonferroni test ($\alpha = 0.05$).

