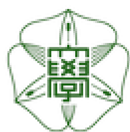
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Title	Enzyme activity modification in adult beetles (Agelastica coerulea) inhabiting birch trees in an ozone-enriched atmosphere
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Citation	Environmental science and pollution research, 25(32), 32675-32683 https://doi.org/10.1007/s11356-018-3243-0
Issue Date	2018-11
Doc URL	http://hdl.handle.net/2115/75984
Rights	The final publication is available at link.springer.com
Type	article (author version)
File Information	Manuscript.pdf

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1 # Enzyme activity modification in adult beetles (*Agelastica coerulea*)
2 # inhabiting birch trees in an ozone-enriched atmosphere

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13

14 ## Abstract

15 Tropospheric ozone (O_3) is a naturally occurring gas in the atmosphere. However, the
16 concentration of O_3 increased in the 20th century. Although the effects of O_3 on vegetation
17 have been extensively studied since the 1950s, limited information exists regarding the
18 effects of O_3 on insect herbivores. In particular, evidence is lacking regarding the effects of
19 O_3 on the biology of insect herbivores. *Agelastica coerulea* (Baly, 1874) is a coleopteran
20 species that grazes on Betulaceae plants. In this study, to investigate the effects of O_3 on *A.*
21 *coerulea* biology for the first time, female adult insects were collected from Japanese white
22 birch trees grown in a Free Air Controlled Exposure System (FACE) in Sapporo, Japan.
23 These beetles inhabited trees exposed either to ambient or to elevated O_3 for 23 days. After

collection, the enzyme activities in the beetles were measured. Elevated O_3 led to a greater total antioxidant activity and lower *α*- and *β*-esterase activities, a phenomenon that may suggest an increased resistance of the beetles to stress. Our results are further discussed with regard to biological and toxicological aspects. Collectively, our findings indicate that total antioxidants and *α*- and *β*-esterase activities can serve as effective O_3 biomarker systems in this beetle species. This adaptive response of the beetle, which was induced by moderate O_3 exposure, should be further tested across generations and for its protection against greater exposure.

Keywords: adaptive response; antioxidants; beetle; enzymes; esterase; hormesis; insect; ozone

Capsule: Ozone treatment increased the total antioxidant activity and decreased the *α* and *β* esterase activity in a leaf beetle.

45 # 1. Introduction

46 Tropospheric ozone (O_3) is a naturally occurring gas in the atmosphere. However, O_3 levels
47 have significantly increased throughout the Northern Hemisphere within the last century and
48 remain elevated (Kalabokas et al. 2017; Nagashima et al. 2017; Sicard et al. 2017; Velasco
49 and Retama 2017; Wang et al. 2017; Solomou et al. 2018). The effects of elevated O_3 levels
50 have been widely studied in plants (Agathokleous et al. 2016a; Fuhrer et al. 2016; Jolivet et al.
51 2016; Li et al. 2017); however, limited data exist for insects (Valkama et al. 2007; Lindroth
52 2010). Global change and O_3-induced alterations in plants may alter the suitability of plants
53 to insects, thus affecting plant-insect interactions in a complex manner (Jøndrup et al. 2002;
54 Valkama et al. 2007; Lindroth 2010; Jamieson et al. 2017). In addition, the *disease triangle*
55 conceptual model, which indicates the interactions among the environment, a plant, and a
56 stressor agent, may be disrupted (Chappelka and Grulke 2016).

57 *Agelastica coerulea* (Baly, 1874) (hereafter referred to as the leaf beetle) is a coleopteran
58 species that grazes on Betulaceae plants (Park et al. 2004; Agathokleous et al. 2017a). This
59 beetle has caused severe damage to Japanese white birch (*Betula platyphylla* var. *japonica*)
60 plants of different ages in the Free Air O_3 Controlled Exposure System (FACE) in Sapporo,
61 Japan (Sakikawa et al. 2014, 2016; Vanderstock et al. 2016). This phenomenon was
62 determined to primarily occur in ambient O_3 sites, whereas damage was far less in elevated
63 O_3 sites. In different laboratory experiments where the leaf beetle was not exposed to O_3,
64 overwintered adults were more attracted to leaves treated with elevated O_3 and grazed on
65 them to a greater extent than leaves in ambient O_3. In contrast, 2nd instar larvae showed no
66 differences between the two leaf types (Agathokleous et al. 2017a). In these experiments,
67 total phenolics and condensed tanning were lower in leaves treated with elevated O_3, whereas
68 leaf mass per area was similar between the two O_3 treatments. In further laboratory assays
69 where the leaf beetle was not exposed to O_3, larvae displayed an increased growth rate,

70 consumption index, and efficiency in the conversion of both ingested and digested food when
71 they were fed leaves treated with elevated O_3 (Abu ElEla et al. 2018). This study suggested
72 that larval performance improved when the larvae were fed O_3-treated leaves, thus abrogating
73 the possibility that adults avoided grazing in elevated O_3 sites to ensure leaf palatability for
74 larvae (Agathokleous et al. 2017a). However, whether O_3 treatment affected the
75 biology/physiology of the adult beetles remains unclear.

76 The activities of transaminase enzymes [glutamic-oxaloacetic transaminase (GOT) and
77 glutamic--pyruvic transaminase (GPT)] and carbohydrate hydrolyzing enzymes (trehalase,
78 invertase, and amylase) can be affected by bioinsecticides (Mead 2000). For example,
79 Murdok et al. (1987) studied the midgut enzymes of various coleopteran pests and found that
80 proteinase activity in the midguts and the redox potential varied with pH and that cysteine
81 proteinases were common digestive enzymes. Similarly, plant defense systems against insect
82 herbivores are mediated in part by enzymes that impair the digestive processes of the insect
83 gut.

84 A close relationship has been found between protein synthesis and levels of transaminases in
85 insects (Wigglesworth 2012). These enzymes provide the building blocks for protein
86 synthesis and may be involved in the synthesis of amino acids during metamorphosis (Osman
87 et al., 2015). Hence, GOT and GPT are used as indicators of protein and amino acid
88 metabolism (Upadhyay et al. 2010). Indeed, transaminases are considered key enzymes in the
89 formation of non-essential amino acids, which if formed inside the body are not taken from
90 outside in the metabolism of nitrogen waste gluconeogenesis (Fagan et al. 2002).
91 Phosphatases are enzymes that hydrolyze phosphorous ester or hydride bonds (O’Brien 1967).
92 Alkaline phosphatase (ALP) is involved in the transphosphorylation reaction.

93 Alpha (α) and beta (β) esterases are detoxifying enzymes that hydrolyze ester bonds and
94 widely respond to environmental stimuli (Hemingway and Karunatne 1998). In addition,
95 esterases are involved in the metabolism of organophosphorus, organochlorine, and several
96 classes of endo- and exogenous compounds, thus playing important roles in insecticide
97 resistance. Indeed, several insecticides contain ester bonds that can be hydrolyzed by esterase
98 activity (Sogorb and Vilanova 2002; Zibaee et al. 2009; Montella et al. 2012). Changes in
99 esterases may affect insect physiology and behavior because important molecules such as
100 pheromones are hydrolyzed by esterases (Montella et al. 2012).

101 Carbohydrates can be utilized for the production of energy and thus are critical for the
102 development of the insect body. The metabolism of carbohydrates is controlled by trehalase,
103 amylase, and invertase enzymes, which play principal roles in insects during the digestion
104 and utilization of carbohydrates (Wigglesworth 2012).

105 The effects of O_3 on physiological performance of insects and particularly on enzymes such
106 as GOT, GPT, LDH, ALP, α and β esterases, invertase, and trehalase, remain unknown. In
107 this study, we aimed to examine the effects of O_3 on adult leaf beetles, with focus on
108 potential alterations in metabolic enzymes. We also explored relationships among response
109 variables and screened response variables as indices for assessing the effects of O_3 on the
110 beetles (i.e., biomarkers) (Strimbu and Tavel 2010).

111 2. Materials and Methods

112 2.1. Insect samples

113 Female adult leaf beetles were collected on July 24th, 2016, from Japanese white birch trees
114 grown in the O_3-FACE system of Sapporo Experimental Forest of Hokkaido University,
115 Japan (43°04' N, 141°20' E, 15 m a.s.l.). Three beetles were randomly sampled from each

116 plot for a total of 9 beetles per O_3 treatment. The sampled beetles were 23 days old (i.e. end
117 of their life as overwintered adults), for a total of 63 days from the egg stage. The duration at
118 the other stages was 3, 24, 2, and 12 days for the egg, larval, pre-pupae, and pupal stages,
119 respectively. Beetle sampling was done in two stages. First, dozens of beetles were examined
120 in each plot as to their age. The identification of the beetle age was based upon three aspects:

121 i) mouthparts: beetles develop chewing mouthparts to feed on leaves. A scale of the size
122 of mandibles and their sharpness was created in relation to age. The more they use,
123 the older beetles are, hence, insects of the same age have the same sharpness of
124 the mandibles;

125 ii) body color: all the beetles are dark but the older ones have darker body;

126 iii) body size: the selected samples for analysis should be also of similar size.

127 A pool of 12 beetles, selected following the identification criteria, was created for each plot.
128 Second, 3 beetles were sampled from the pool of each plot for biochemical analyses.

129 This FACE system, which has been in operation since 2014, has six ring units (plots), three
130 of which contain ambient air (AOZ) and three that contain ambient air enriched with O_3
131 (EOZ). The target O_3 concentration in the EOZ was 70 nmol mol^{-1} during the daytime (>70
132 μmol m^{-2}s^{-1}: light compensation point of tested plants; Koike, 1988). O_3 was monitored in
133 each EOZ plot by SM70 Fixed Ozone Monitors (Aeroqual Ltd., Auckland, NZ) and in one
134 AOZ plot using a Model 202 O_3 monitor (2B Technologies, Boulder CO, USA). The mean 10
135 h O_3 concentration was 60 nmol mol^{-1} in 2014 (August 15 to October 26) and 72 nmol mol^{-1}
136 in 2015 (April 24 to October 26) in the EOZ plots, and 20 nmol mol^{-1} in 2014 and 34 nmol
137 mol^{-1} in 2015 in the AOZ plots. In 2016, O_3 treatment started on May 18, and the 10 h mean
138 O_3 concentration (07:00–17:00) in the EOZ plots was 63.5 nmol mol^{-1} (May 18 to July 24).

139 The monitoring of the ambient O_3 concentration started from June 1. The 10 h ambient O_3
140 concentration was 16.93 nmol mol^{-1} (May 18 to July 24).

141 Details on the FACE system, O_3 exposure, and meteorological conditions of the previous
142 years have been previously described (Agathokleous et al. 2016b, 2017b).

143 **2.2. Sample analyses**

144 Insects were homogenized for biochemical analysis in a chilled glass Teflon tissue
145 homogenizer (ST-2 Mechanic-Preczyina, Poland). After homogenization, supernatants were
146 kept in a deep freezer at −20°C until use for biochemical assays.

147 The insects were prepared as described by Amin (1998). They were homogenized in distilled
148 water (50 mg mL^{-1}). Homogenates were centrifuged at 8000 rpm for 15 min at 2°C. The
149 deposits were discarded, and the supernatants, referred to as enzyme extracts, were stored for
150 less than one week before analysis.

151 Antioxidants were measured using biodiagnostic kit No. TA 2513. Antioxidants in the sample
152 react with a known quantity of exogenous H_2O_2 of which they eliminate a certain amount.
153 The residual H_2O_2 was determined colorimetrically by an enzymatic reaction which involves
154 the conversion of 3,5-Dichloro-2-benzensulfonate to a colored product read at 505 nm. Total
155 carbohydrates were estimated in acid extract of sample by the phenol-sulfuric acid reaction of
156 DuBois et al. (1956). The absorbance was measured at 490 nm against blank. Total proteins
157 were determined by the method of Bradford (I976), using Coomassie Brilliant blue G-250 for
158 dye. The absorbance was measured at 595 nm against blank prepared from 1 ml of phosphate
159 buffer and 5 ml protein reagent.

160 Alpha- and *β*-esterases were determined according to Van Asperen (I962), using *α*-naphthyl
161 acetate or *β*-naphthyl acetate as substrates. The reaction mixture consisted of 5 ml substrate

162 solution ($3x10^{-4}$ M α-or β-naphthylacetate, 1% acetone, and 0.1 M phosphate buffer, pH 7)
163 and 20 µl of homogenate. The mixture was incubated for 15 min at 27˚C, and 1 ml of diazo
164 blue color reagent was added; the reagent was prepared by mixing 2 parts of 1% diazo blue B
165 and 5 parts of 5% sodium lauryl sulfate. The absorbance was measured at 600 or 555 nm for
166 α- and β-naphthol produced from hydrolysis of the substrate.

167 GOT and GPT were determined colorimetrically according to the method of Reitman and
168 Frankle (1957). The optical density of the produced brown color was measured at 520 nm
169 after 5 minutes, using a spectrophotometer. The method of LDH determination was derived
170 from the formulation recommended by the German Society for Clinical Chemistry (1970).
171 Lactate dehydrogenase catalyzes the conversion of pyruvate to lactate, and NADH is
172 oxidized to NAD during the process. The rate of decrease in NADH is proportional to the
173 LDH activity.

174 Acid and alkaline phosphatases were determined according to the method described by
175 Powell and Smith (1954). In this method, the phenol released by enzymatic hydrolysis of
176 disodium phenylphosphate reacts with 4-aminoantipyrine, and by the addition of potassium
177 ferricyanide, a characteristic brown color is produced. The absorbance was measured at 510
178 nm. The enzyme activity is expressed by unit (U), where 1 unit will hydrolyze 1.0 umole of
179 p-nitophenyl phosphate per minute at 37 C^{o}, and pH 10.4 and 4.8 for alkaline and acid
180 phosphatases, respectively.

181 Digestive enzymes were determined according to the modifications of Amin (1998) to the
182 method described by Ishaaya and Swirski (1976). Generally, 20 µl of diluted enzyme solution
183 was incubated for 10 min at 30 ˚C with 250 µl of 4% sucrose for invertase activity or 3%
184 trehalose solution for trehalase activity, and 230 ul phosphate buffer (pH 5.4, 0.1 M). The
185 reaction was ceased by adding 250 µl DNS reagent to each tube in boiling water for 5 min.

186 Samples were cooled, diluted with 2.5 ml H_2O, and the absorbance was measured at 550 nm.
187 Glucose was used as a standard. Appropriate dilutions of enzyme supernatant were used to
188 obtain a linear production of glucose equivalents.

189 Bovine albumin standard was purchased from Stanbio laboratory (Texas, USA). Coomassie
190 brilliant blue G-250 was purchased from Sigma (Sigma Chemical Co.). P-nitroanisole (purity
191 97%) was obtained from Ubichem Ltd. (Hampshire, England) and nicotinamide adenine
192 dinucleotide phosphate (reduced form NADPH) was from BDH Chemicals Ltd. (Poole,
193 England). The rest of the chemicals were of high quality and purchased from commercial
194 local companies. A double beam ultraviolet/visible spectrophotometer (Spectronic 1201,
195 Milton Roy Co., USA) was used to measure the absorbance of colored substances and
196 metabolic compounds.

197 **2.3. Statistical analysis**

198 The cut-off for statistically significant results was set at an α level of 0.05. Because of the *a*
199 *priori* planned comparisons, the data from each response variable were averaged per
200 experimental unit to provide 3 real replicates, and subjected to simple contrasts of Least
201 Squares means (Agathokleous et al. 2016b). The single degree of freedom (k-1) was
202 partitioned to the contrast (a) AOZ vs. EOZ.

203 For statistically significant linear contrasts, the effect magnitude of EOZ was calculated using
204 a bias-corrected Cohen's δ (Hedges and Olkin 1985; Cohen 1988), as described previously
205 (Agathokleous et al. 2016c). The effects were converted into percentile gain, which appeared
206 in the experimental condition using the Cohen's U_3 index (Cohen 1977); δ was converted to
207 the overlapping coefficient (OVL) (Reiser and Faraggi 1999). The effect magnitude was
208 arbitrarily classified as neutral [δ = [0.00, 0.50)], small [δ = [0.50, 1.50)], moderate [δ =
209 [1.50–3.00)], or large (δ = 3.00+) (Cohen 1988; Agathokleous et al., 2016b). Absolute δ

210 values in the interval [0.50–1.50] indicated educational significance, whereas δ values >1.50
211 indicated clinical significance (Wolf 1986; Agathokleous et al. 2016b).

212 To explore the relationships between the response variables and to screen the response
213 variables as indices for assessing the effects of O_3, the data of the response variables were
214 transformed to *T*-scores, with a mean of 50 and an SD of 10 (Agathokleous et al. 2016d), and
215 subjected to principal component analysis (PCA) with varimax rotation (Kaiser 1958; Abdi
216 and Williams 2010). The null hypothesis of correlations between variables was tested by
217 simple linear regression analysis. Regarding correlations, bivariate correlation (r) values
218 within the arbitrary segments [0.00, 0.10), [0.10, 0.30), [0.30, 0.50), [0.50, 0.70), [0.70, 0.90),
219 and 0.90+ indicated correlations of trivial, low, moderate, large, very large, and nearly perfect
220 magnitude, respectively (Hopkins 2000).

221 The software used for processing and to statistically analyze the data included MS EXCEL
222 2010 (Microsoft ©) and STATISTICA v.10 (StatSoft Inc. ©).

223
3. Results & Discussion

224 Insects in the EOZ group had 124% greater total antioxidants than insects in the AOZ group
225 (Fig 1*A*). In addition, insects in the AOZ group had 184% and 138% greater α and β esterases
226 than insects in the EOZ group (Fig 2). EOZ had a moderate effect on total antioxidants (δ =
227 1.80, U_3 = 0.96, OVL = 0.37), α esterases (δ = −2.71, U_3 = 1.00, OVL = 0.17), and β
228 esterases (δ = −2.16, U_3 = 0.98, OVL = 0.28), which was of both practical and clinical
229 significance. Increased antioxidants may contribute to improving the performance of insects
230 under stress by decreasing damage to lipids and proteins induced by free radicals (Tapia et al.
231 2006; López-Martínez et al. 2012, 2016). The EOZ-induced decrease in esterases may be
232 interpreted as an inhibitory effect of O_3 on the synthesis of α and β esterases (Kurappasamy et
233 al. 2001). However, this may not be the case. Insects can achieve resistance through the

234 modification of enzyme structures to increase the capacity to metabolize harmful products. In
235 this case, the decreased α and β esterase activity due to the EOZ treatment may indicate
236 increased resistance (Wool and Greenberg 1990), along with an enhanced capacity to
237 hydrolyze harmful substances. This, along with a lower hydrolysis of substrates that drive the
238 activity of esterases, is in accordance with the "mutant ali-esterase" theory (Oppenoorth and
239 van Asperen 1960). Hence, esterase activity may serve as a biomarker of resistance to stress,
240 where lower esterase activity is associated with greater resistance (Wool and Greenberg
241 1990).

242 Insects may experience stress indirectly by consuming harmful materials in O_3-affected
243 leaves or directly by breathing O_3 included in the air taken from the tracheae (a tubes
244 network). This study aimed at investigative O_3 effects on the beetle as a sum of direct and
245 indirect effects; insects would experience both direct and indirect effects in an O_3-polluted
246 environment. Hence, this study does not provide the opportunity to separate the direct and
247 indirect effects of O_3 (Telesnicki 2015, 2018). However, previous bioassays, where this
248 beetle was not directly exposed to O_3, suggest that EOZ leaves not only were not harmful but
249 were often preferred over AOZ leaves by the beetles. In addition, the beetles fed with EOZ
250 leaves often had improved nutritional performance compared with beetles fed with AOZ
251 leaves (Agathokleous et al. 2017a; Abu ElEla et al. 2018). Hence, the present study may
252 suggest for the first time a direct biological effect of elevated O_3 on this beetle. The Japanese
253 environmental quality standard for O_3 hourly values is set at 0.06 ppm (Ministry of the
254 Environment, Government of Japan; https://www.env.go.jp/en/air/aq/aq.html), which is
255 similar to the O_3 concentration in the EOZ. Based on Japanese and other worldwide (e.g., U.S.
256 EPA) standards, which are set according to the literature using several animal models,
257 adverse EOZ-induced effects on beetles would not be expected to occur at O_3 exposures
258 lower than the standards. Rather, increased oxidative stress below the toxicological threshold

259 may promote health in animal models (Ristow and Schmeisser 2011). In fact, it is known that
260 O_3 can induce the up-regulation of antioxidants in animal models, including humans, which
261 are of an adaptive nature, resulting in potential effects that are beneficial to health (Bocci
262 2006, 2007, 2012). These effects may also protect against a subsequent more massive
263 environmental threat, a phenomenon called *environmental conditioning* (Calabrese et al.
264 2016a,b; Agathokleous 2018). Adaptive responses should be further tested over time and
265 across generations due to potential transgenerational hormetic mechanisms (Calabrese and
266 Mattson 2017; Agathokleous 2018; Agathokleous et al. 2018).

267 Insects in the AOZ and EOZ group did not significantly differ in terms of total carbohydrates
268 (Fig 1*B*), total proteins (Fig 1*C*), GOT (Fig 3*A*), GPT (Fig 3*B*), LDH (Fig 3*C*), ALP (Fig 4*A*),
269 invertase (Fig 4*B*), and trehalase (Fig 4*C*). In several response variables, there were large
270 proportional differences between the AOZ and EOZ groups; however, they displayed a large
271 relative standard deviation (RSD). For example, ALP was 1.87 times greater in insects in the
272 EOZ group than in the AOZ group; however, the RSD in the EOZ group was 56.9% vs. 9.8%
273 in the AOZ group.

274 The relationships among response variables were also examined. With regards to PCA, the
275 first three factors, F1, F2, and F3, explained 36.35%, 28.97%, and 24.74% of the total
276 variance, respectively, for a total of 90% (Fig 5). The three response variables with a
277 significant EOZ effect, i.e., the total antioxidants and α and β esterases, had a high loading on
278 the major axis PC1 and explained 47.9% of the variance in this component (Table 1). Total
279 antioxidants were negatively correlated with α and β esterases (Fig 5), suggesting that an
280 increase in total antioxidants is accompanied by a decrease in α and β esterases. Furthermore,
281 the high loading of total antioxidants, and α and β esterases on PC1, reflected a high
282 correlation of these response variables with PC1, suggesting that, along with total
283 antioxidants, α and β esterases may be considered as an interesting biomarker of the effects of

O_3 on these insects when the effects are not confounded by other environmental stresses such as drought.

Total antioxidants displayed an anticorrelation of a very large magnitude with α and β esterases, and a positive correlation of very large magnitude with LDH (Table 2). This may indicate that esterase reduction is associated with LDH enhancement along with increased glycolytic capacity. It is important that LDH in animal models display a biphasic dose response to stress (Diamantino et al. 2001), i.e., hormesis, which suggests the stimulation of LDH at low exposure levels and the inhibition of LDH at high levels of exposure (Agathokleous 2018). Total antioxidants were negatively correlated with total carbohydrates; however, the *P*-value was not significant (P = 0.060). Total carbohydrates displayed a positive correlation of very large magnitude with total proteins and β esterases. ALP was negatively correlated with invertase. All other correlations were not significant.

4. Conclusions

- EOZ increased the total antioxidant activity and decreased the α and β esterase activity.
- The results of this study indicate EOZ-induced oxidative stress in this beetle.
- Higher antioxidant capacity and lower esterase capacity suggest increased resistance along with a health-promoting capability through the prevention of the accumulation of harmful substances produced during stress, such as reactive oxygen species.
- Total antioxidants and esterases (α and β) can be utilized as an effective O_3 biomarker system in this beetle.
- O_3-induced biological responses of the beetle require further experimentations over time and across generations.

307 • A further challenging task would be to test whether potential adaptive responses of
308 the beetle to sub-lethal O_3-induced stress result in less harm following larger stress.
309
310 **Acknowledgements**: The first author wishes to thank the Mission Sector of Higher
311 Education of the Government of Egypt and the Culture Office of the Embassy of Egypt in
312 Tokyo, Japan for support and funding during the research in Hokkaido University, Japan. E.
313 Agathokleous is an International Research Fellow (ID No: P17102) of the Japan Society for
314 the Promotion of Science (JSPS).
315
316 **Funding**: This study was funded in part by a Grant–in–Aid from JSPS through Type B
317 program (Kaken grant numbers 26292075 and 16K14932, to T. Koike). JSPS is a non-profit,
318 independent administrative institution.
319

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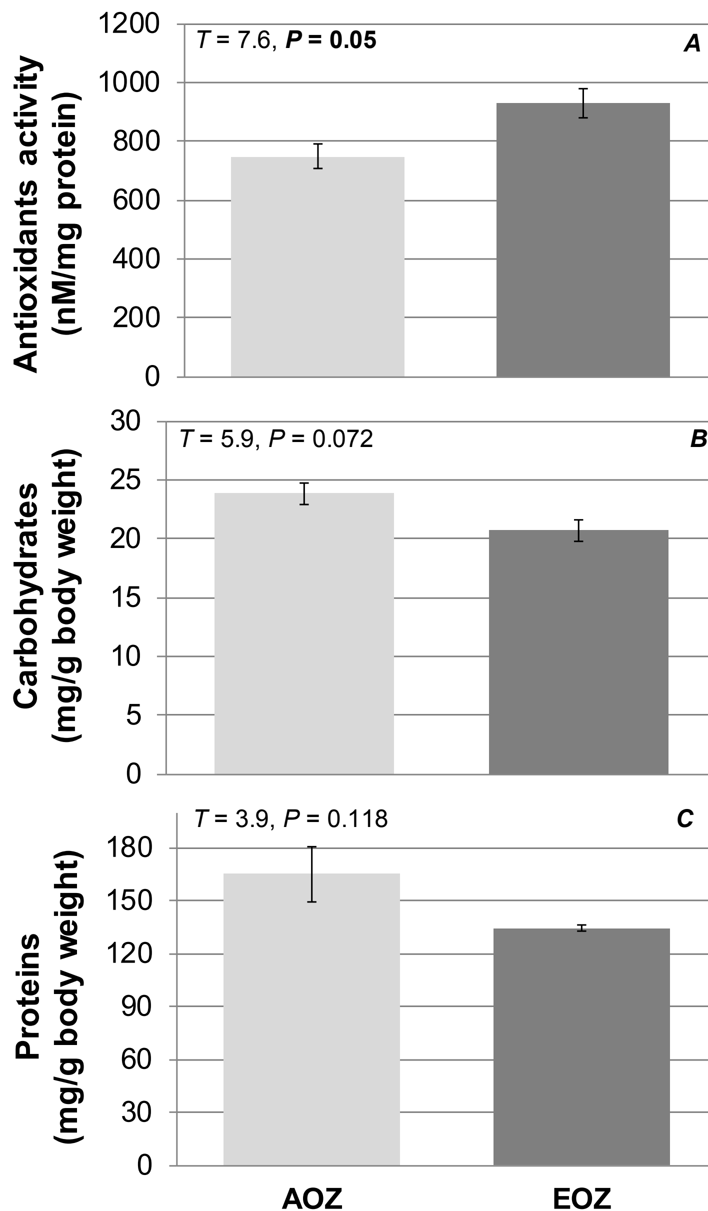
510 Fig 1

511 **Fig. 1** The means (±SE) of total antioxidant activity (*A*), total carbohydrate content (*B*), and
512 total protein content (*C*) in adult insects of *Agelastica coerulea* in ambient (AOZ) or elevated
513 (EOZ) O_3 conditions. Data were statistically analyzed by the simple contrast AOZ *vs.* EOZ (n
514 = 3). *P*-values marked with bold indicate statistical significance at an α level of 0.05.



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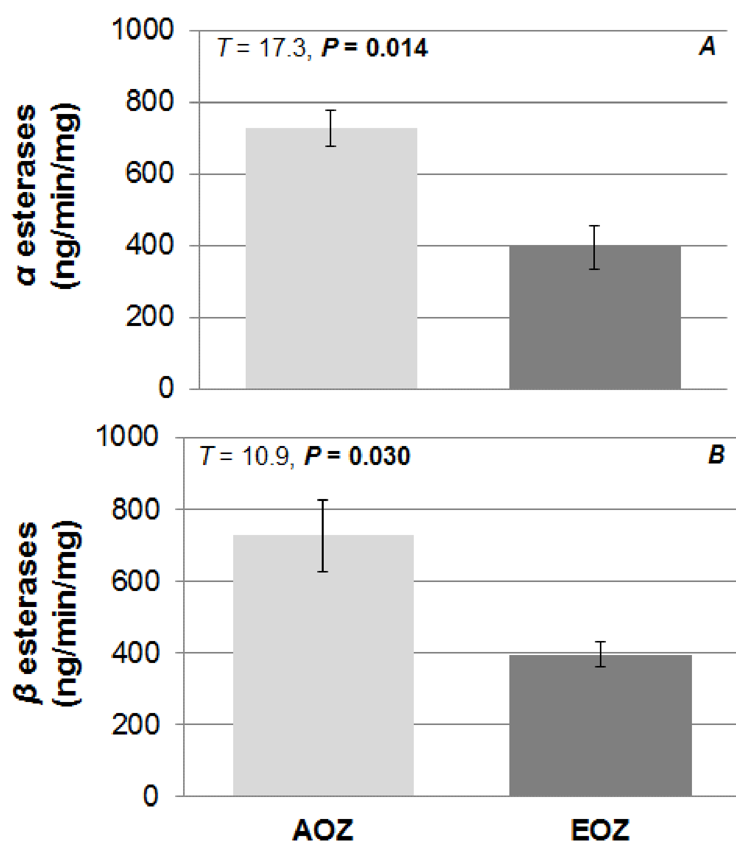
517 Fig 2

518 **Fig. 2** The means (±SE) of alpha (*A*) and beta (*B*) esterases in adult insects of *Agelastica*
519 *coerulea* in ambient (AOZ) or elevated (EOZ) O_3 conditions. The units are ng *α*-naphthol
520 min^{-1} mg^{-1} protein for alpha esterases and ng *β*-naphthol min^{-1} mg^{-1} protein for beta esterases.
521 Data were statistically analyzed by the simple contrast AOZ *vs.* EOZ (*n* = 3). *P*-values
522 marked with bold indicate statistical significance at an *α* level of 0.05.



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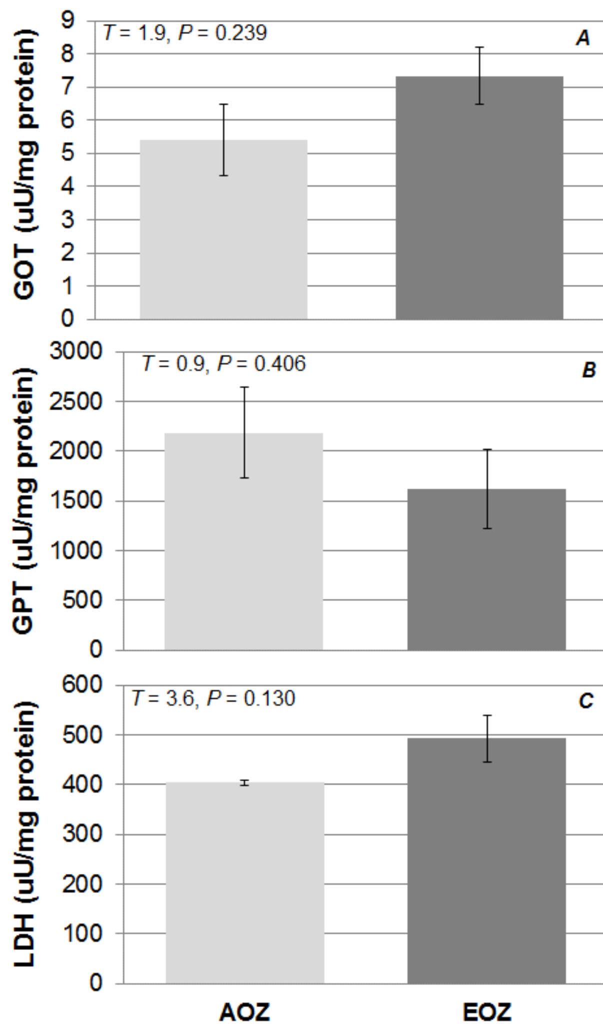
525 Fig 3

526 **Fig. 3** The means (±SE) of glutamic-oxaloacetic transaminase, GOT (*A*), glutamic--pyruvic 527 transaminase, GPT (*B*), and lactate dehydrogenase, LDH (*C*) in adult insects of *Agelastica* 528 *coerulea* in ambient (AOZ) or elevated (EOZ) O_3 conditions. Data were statistically analyzed 529 by the simple contrast AOZ *vs.* EOZ (n = 3), at an α level of 0.05.



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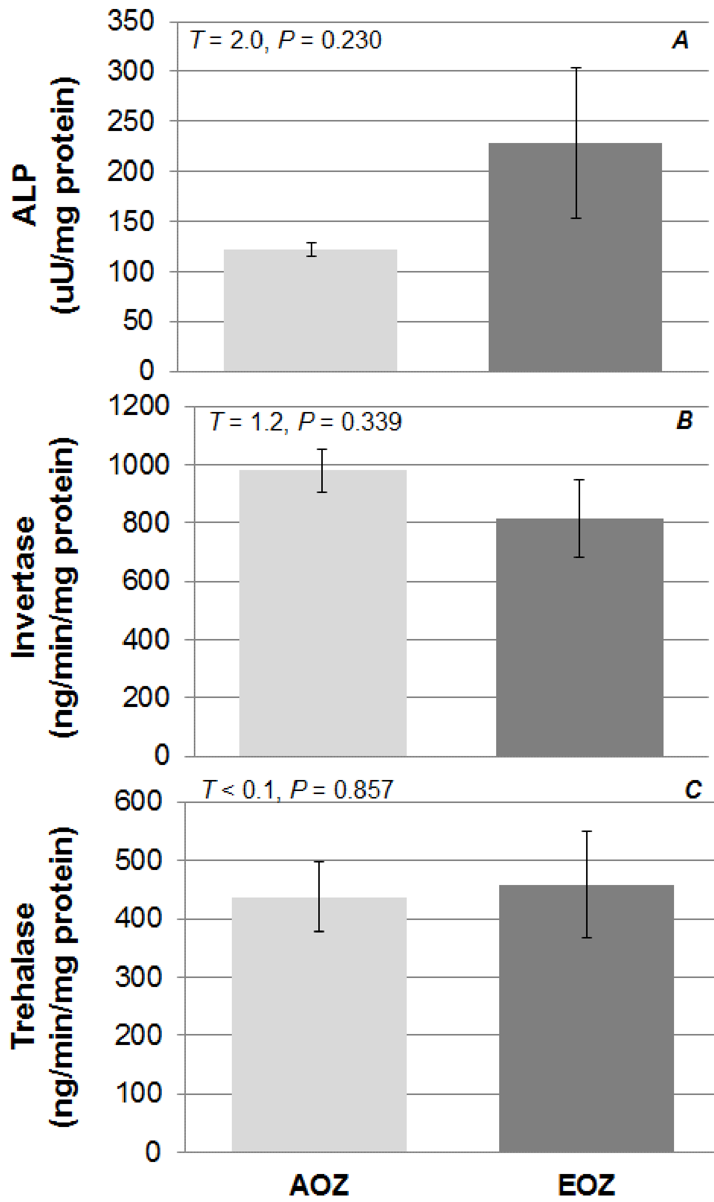
532 Fig 4

533 **Fig. 4** The means (±SE) of alkaline phosphatase, ALP (*A*), invertase (*B*), and trehalase (*C*) in

534 adult insects of *Agelastica coerulea* in ambient (AOZ) or elevated (EOZ) O_3 conditions. Data

535 were statistically analyzed by the simple contrast AOZ *vs.* EOZ (n = 3), at an α level of 0.05.



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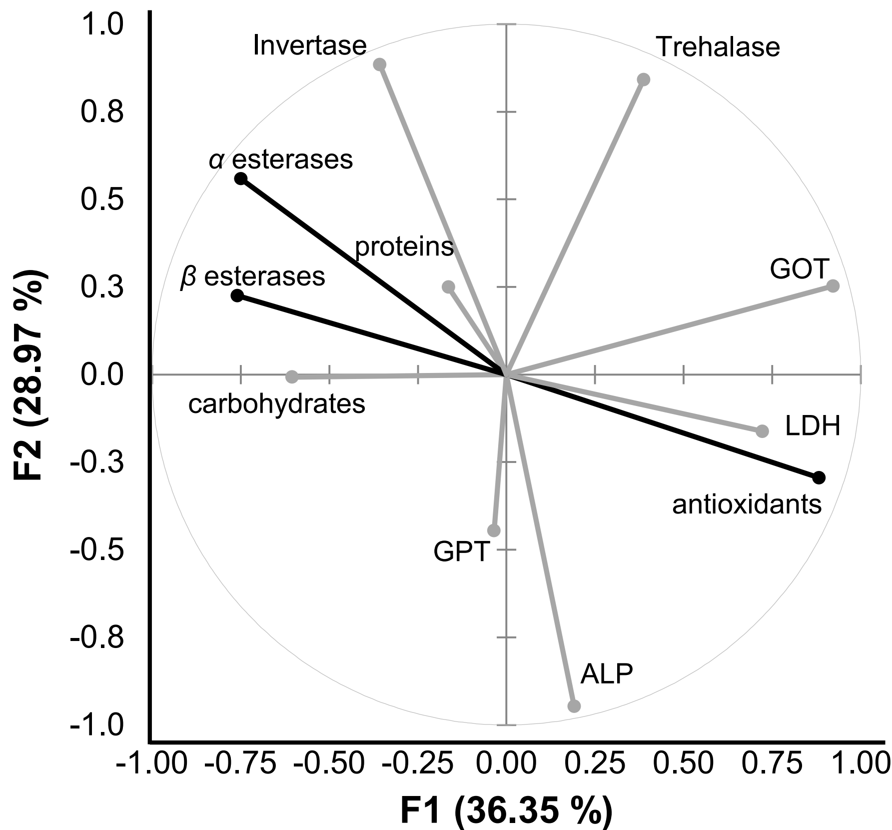
538 Fig 5

539 **Fig. 5** Biplot of the PCA ordination with varimax rotation based on T-scores of the response
540 variables. Response variables on which elevated O_3 had a statistically significant effect are
541 represented by black lines and markers, whereas those on which elevated O_3 had no
542 statistically significant effect are represented by gray lines and markers. The response
543 variables were: alpha esterases (α esterases), alkaline phosphatase (ALP), beta esterases (β
544 esterases), glutamic-oxaloacetic transaminase (GOT), glutamic--pyruvic transaminase (GPT),
545 invertase, lactate dehydrogenase (LDH), total antioxidants, total carbohydrates, total proteins,
546 and trehalase.



547

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549 Table 1

550 **Table 1** The factor loading and the contribution of variable (%) after principal components 551 analysis based on T-scores of the response variables. Response variables on which elevated 552 O_3 had a statistically significant effect are represented by bold text, whereas those on which 553 elevated O_3 had no statistically significant effect are represented by non-bold text. The 554 response variables were: alpha esterases (α esterases), alkaline phosphatase (ALP), beta 555 esterases (β esterases), glutamic-oxaloacetic transaminase (GOT), glutamic--pyruvic 556 transaminase (GPT), invertase, lactate dehydrogenase (LDH), total antioxidants, total 557 carbohydrates, total proteins, and trehalase.

	Factor loadings			**Contribution of the variables (%)**		
	F1	**F2**	**F3**	**F1**	**F2**	**F3**
***α* esterases**	**−0.750**	**0.559**	**0.178**	**14.051**	**9.810**	**1.160**
ALP	0.191	−0.947	−0.129	0.916	28.114	0.613
***β* esterases**	**−0.760**	**0.225**	**0.480**	**14.443**	**1.594**	**8.452**
GOT	0.922	0.253	0.029	21.277	2.003	0.031
GPT	−0.035	−0.446	0.844	0.030	6.228	26.182
Invertase	−0.357	0.884	−0.224	3.195	24.532	1.851
LDH	0.723	−0.162	−0.326	13.064	0.825	3.905
Total antioxidants	**0.882**	**−0.294**	**−0.285**	**19.449**	**2.718**	**2.984**
Total carbohydrates	−0.605	−0.007	0.770	9.153	0.002	21.764
Total proteins	−0.164	0.265	0.946	0.671	1.953	32.887
Trehalase	0.387	0.842	0.068	3.753	22.222	0.170

558

Table 2

Table 2 Bivariate correlation (r) values for pairs of response variables. Bold values were different from 0 with a significance level $\alpha = 0.05$, whereas non-bold values showed non-statistical significance at an α level of 0.05. The response variables were: alpha esterases (α esterases), alkaline phosphatase (ALP), beta esterases (β esterases), glutamic-oxaloacetic transaminase (GOT), glutamic--pyruvic transaminase (GPT), invertase, lactate dehydrogenase (LDH), total antioxidants, total carbohydrates, total proteins, and trehalase.

Variables	α esterases	ALP	β esterases	GOT	GPT	Invertase	LDH	Total antioxidants	Total carbohydrates	Total proteins	Trehalase
α esterases	1	−0.7610	0.7205	−0.5069	0.0155	0.7597	−0.7144	**−0.8284**	0.5266	0.4253	0.0818
ALP	−0.7610	1	−0.4021	−0.0737	0.2396	**−0.9138**	0.3086	0.4384	−0.1623	−0.3953	−0.6482
β esterases	0.7205	−0.4021	1	−0.7387	0.2954	0.3904	−0.5396	**−0.8478**	**0.8445**	0.6779	−0.0285
GOT	−0.5069	−0.0737	−0.7387	1	−0.1018	−0.1397	0.4530	0.7040	−0.5442	−0.0954	0.5497
GPT	0.0155	0.2396	0.2954	−0.1018	1	−0.5253	−0.2121	−0.0835	0.6135	0.6967	−0.4408
Invertase	0.7597	**−0.9138**	0.3904	−0.1397	−0.5253	1	−0.2454	−0.4648	0.0033	0.0848	0.5331
LDH	−0.7144	0.3086	−0.5396	0.4530	−0.2121	−0.2454	1	**0.8670**	−0.7092	−0.3927	0.1003
Total antioxidants	**−0.8284**	0.4384	**−0.8478**	0.7040	−0.0835	−0.4648	**0.8670**	1	−0.7921	−0.4680	0.0036
Total carbohydrates	0.5266	−0.1623	**0.8445**	−0.5442	0.6135	0.0033	−0.7092	−0.7921	1	**0.8203**	−0.1126
Total proteins	0.4253	−0.3953	0.6779	−0.0954	0.6967	0.0848	−0.3927	−0.4680	**0.8203**	1	0.2060
Trehalase	0.0818	−0.6482	−0.0285	0.5497	−0.4408	0.5331	0.1003	0.0036	−0.1126	0.2060	1