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

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## Abstract

Tropospheric ozone (O<sub>3</sub>) is a naturally occurring gas in the atmosphere. However, the concentration of O<sub>3</sub> increased in the 20<sup>th</sup> century. Although the effects of O<sub>3</sub> on vegetation have been extensively studied since the 1950s, limited information exists regarding the effects of O<sub>3</sub> on insect herbivores. In particular, evidence is lacking regarding the effects of O<sub>3</sub> on the biology of insect herbivores. *Agelastica coerulea* (Baly, 1874) is a coleopteran species that grazes on Betulaceae plants. In this study, to investigate the effects of O<sub>3</sub> on *A. coerulea* biology for the first time, female adult insects were collected from Japanese white birch trees grown in a Free Air Controlled Exposure System (FACE) in Sapporo, Japan. These beetles inhabited trees exposed either to ambient or to elevated O<sub>3</sub> for 23 days. After

collection, the enzyme activities in the beetles were measured. Elevated O<sub>3</sub> led to a greater total antioxidant activity and lower  $\alpha$ - and  $\beta$ -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  $\alpha$ - and  $\beta$ -esterase activities can serve as effective O<sub>3</sub> biomarker systems in this beetle species. This adaptive response of the beetle, which was induced by moderate O<sub>3</sub> 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  $\alpha$  and  $\beta$  esterase activity in a leaf beetle.

## 1. Introduction

Tropospheric ozone (O<sub>3</sub>) is a naturally occurring gas in the atmosphere. However, O<sub>3</sub> levels have significantly increased throughout the Northern Hemisphere within the last century and remain elevated (Kalabokas et al. 2017; Nagashima et al. 2017; Sicard et al. 2017; Velasco and Retama 2017; Wang et al. 2017; Solomou et al. 2018). The effects of elevated O<sub>3</sub> levels have been widely studied in plants (Agathokleous et al. 2016a; Fuhrer et al. 2016; Jolivet et al. 2016; Li et al. 2017); however, limited data exist for insects (Valkama et al. 2007; Lindroth 2010). Global change and O<sub>3</sub>-induced alterations in plants may alter the suitability of plants to insects, thus affecting plant-insect interactions in a complex manner (Jøndrup et al. 2002; Valkama et al. 2007; Lindroth 2010; Jamieson et al. 2017). In addition, the *disease triangle* conceptual model, which indicates the interactions among the environment, a plant, and a stressor agent, may be disrupted (Chappelka and Grulke 2016).

*Agelastica coerulea* (Baly, 1874) (hereafter referred to as the leaf beetle) is a coleopteran species that grazes on Betulaceae plants (Park et al. 2004; Agathokleous et al. 2017a). This beetle has caused severe damage to Japanese white birch (*Betula platyphylla* var. *japonica*) plants of different ages in the Free Air O<sub>3</sub> Controlled Exposure System (FACE) in Sapporo, Japan (Sakikawa et al. 2014, 2016; Vanderstock et al. 2016). This phenomenon was determined to primarily occur in ambient O<sub>3</sub> sites, whereas damage was far less in elevated O<sub>3</sub> sites. In different laboratory experiments where the leaf beetle was not exposed to O<sub>3</sub>, overwintered adults were more attracted to leaves treated with elevated O<sub>3</sub> and grazed on them to a greater extent than leaves in ambient O<sub>3</sub>. In contrast, 2<sup>nd</sup> instar larvae showed no differences between the two leaf types (Agathokleous et al. 2017a). In these experiments, total phenolics and condensed tanning were lower in leaves treated with elevated O<sub>3</sub>, whereas leaf mass per area was similar between the two O<sub>3</sub> treatments. In further laboratory assays where the leaf beetle was not exposed to O<sub>3</sub>, larvae displayed an increased growth rate,

consumption index, and efficiency in the conversion of both ingested and digested food when they were fed leaves treated with elevated O<sub>3</sub> (Abu ElEla et al. 2018). This study suggested that larval performance improved when the larvae were fed O<sub>3</sub>-treated leaves, thus abrogating the possibility that adults avoided grazing in elevated O<sub>3</sub> sites to ensure leaf palatability for larvae (Agathokleous et al. 2017a). However, whether O<sub>3</sub> treatment affected the biology/physiology of the adult beetles remains unclear.

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

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

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

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

The effects of O<sub>3</sub> on physiological performance of insects and particularly on enzymes such as GOT, GPT, LDH, ALP,  $\alpha$  and  $\beta$  esterases, invertase, and trehalase, remain unknown. In this study, we aimed to examine the effects of O<sub>3</sub> on adult leaf beetles, with focus on potential alterations in metabolic enzymes. We also explored relationships among response variables and screened response variables as indices for assessing the effects of O<sub>3</sub> on the beetles (i.e., biomarkers) (Strimbu and Tavel 2010).

## 2. Materials and Methods

### 2.1. Insect samples

Female adult leaf beetles were collected on July 24<sup>th</sup>, 2016, from Japanese white birch trees grown in the O<sub>3</sub>-FACE system of Sapporo Experimental Forest of Hokkaido University, Japan (43°04' N, 141°20' E, 15 m a.s.l.). Three beetles were randomly sampled from each

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

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

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

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

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

This FACE system, which has been in operation since 2014, has six ring units (plots), three of which contain ambient air (AOZ) and three that contain ambient air enriched with O<sub>3</sub> (EOZ). The target O<sub>3</sub> concentration in the EOZ was 70 nmol mol<sup>-1</sup> during the daytime (>70 μmol m<sup>-2</sup>s<sup>-1</sup>: light compensation point of tested plants; Koike, 1988). O<sub>3</sub> was monitored in each EOZ plot by SM70 Fixed Ozone Monitors (Aeroqual Ltd., Auckland, NZ) and in one AOZ plot using a Model 202 O<sub>3</sub> monitor (2B Technologies, Boulder CO, USA). The mean 10 h O<sub>3</sub> concentration was 60 nmol mol<sup>-1</sup> in 2014 (August 15 to October 26) and 72 nmol mol<sup>-1</sup> in 2015 (April 24 to October 26) in the EOZ plots, and 20 nmol mol<sup>-1</sup> in 2014 and 34 nmol mol<sup>-1</sup> in 2015 in the AOZ plots. In 2016, O<sub>3</sub> treatment started on May 18, and the 10 h mean O<sub>3</sub> concentration (07:00–17:00) in the EOZ plots was 63.5 nmol mol<sup>-1</sup> (May 18 to July 24).

The monitoring of the ambient O<sub>3</sub> concentration started from June 1. The 10 h ambient O<sub>3</sub> concentration was 16.93 nmol mol<sup>-1</sup> (May 18 to July 24).

Details on the FACE system, O<sub>3</sub> exposure, and meteorological conditions of the previous years have been previously described (Agathokleous et al. 2016b, 2017b).

## **2.2. Sample analyses**

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

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

Antioxidants were measured using biodiagnostic kit No. TA 2513. Antioxidants in the sample react with a known quantity of exogenous H<sub>2</sub>O<sub>2</sub> of which they eliminate a certain amount. The residual H<sub>2</sub>O<sub>2</sub> was determined colorimetrically by an enzymatic reaction which involves the conversion of 3,5-Dichloro-2-benzensulfonate to a colored product read at 505 nm. Total carbohydrates were estimated in acid extract of sample by the phenol-sulfuric acid reaction of DuBois et al. (1956). The absorbance was measured at 490 nm against blank. Total proteins were determined by the method of Bradford (1976), using Coomassie Brilliant blue G-250 for dye. The absorbance was measured at 595 nm against blank prepared from 1 ml of phosphate buffer and 5 ml protein reagent.

Alpha- and  $\beta$ -esterases were determined according to Van Asperen (1962), using  $\alpha$ -naphthyl acetate or  $\beta$ -naphthyl acetate as substrates. The reaction mixture consisted of 5 ml substrate



solution ( $3 \times 10^{-4}$  M  $\alpha$ -or  $\beta$ -naphthylacetate, 1% acetone, and 0.1 M phosphate buffer, pH 7) and 20  $\mu$ l of homogenate. The mixture was incubated for 15 min at 27°C, and 1 ml of diazo blue color reagent was added; the reagent was prepared by mixing 2 parts of 1% diazo blue B and 5 parts of 5% sodium lauryl sulfate. The absorbance was measured at 600 or 555 nm for  $\alpha$ - and  $\beta$ -naphthol produced from hydrolysis of the substrate.

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

Acid and alkaline phosphatases were determined according to the method described by Powell and Smith (1954). In this method, the phenol released by enzymatic hydrolysis of disodium phenylphosphate reacts with 4-aminoantipyrine, and by the addition of potassium ferricyanide, a characteristic brown color is produced. The absorbance was measured at 510 nm. The enzyme activity is expressed by unit (U), where 1 unit will hydrolyze 1.0  $\mu$ mole of p-nitrophenyl phosphate per minute at 37 °C, and pH 10.4 and 4.8 for alkaline and acid phosphatases, respectively.

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

Samples were cooled, diluted with 2.5 ml H<sub>2</sub>O, and the absorbance was measured at 550 nm. Glucose was used as a standard. Appropriate dilutions of enzyme supernatant were used to obtain a linear production of glucose equivalents.

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

### 2.3. Statistical analysis

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

For statistically significant linear contrasts, the effect magnitude of EOZ was calculated using a bias-corrected Cohen's  $\delta$  (Hedges and Olkin 1985; Cohen 1988), as described previously (Agathokleous et al. 2016c). The effects were converted into percentile gain, which appeared in the experimental condition using the Cohen's U<sub>3</sub> index (Cohen 1977);  $\delta$  was converted to the overlapping coefficient (OVL) (Reiser and Faraggi 1999). The effect magnitude was arbitrarily classified as neutral [ $\delta$  = [0.00, 0.50)], small [ $\delta$  = [0.50, 1.50)], moderate [ $\delta$  = [1.50–3.00)], or large ( $\delta$  = 3.00+) (Cohen 1988; Agathokleous et al., 2016b). Absolute  $\delta$

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

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

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

### 3. Results & Discussion

Insects in the EOZ group had 124% greater total antioxidants than insects in the AOZ group (Fig 1A). In addition, insects in the AOZ group had 184% and 138% greater  $\alpha$  and  $\beta$  esterases than insects in the EOZ group (Fig 2). EOZ had a moderate effect on total antioxidants ( $\delta = 1.80$ ,  $U_3 = 0.96$ ,  $OVL = 0.37$ ),  $\alpha$  esterases ( $\delta = -2.71$ ,  $U_3 = 1.00$ ,  $OVL = 0.17$ ), and  $\beta$  esterases ( $\delta = -2.16$ ,  $U_3 = 0.98$ ,  $OVL = 0.28$ ), which was of both practical and clinical significance. Increased antioxidants may contribute to improving the performance of insects under stress by decreasing damage to lipids and proteins induced by free radicals (Tapia et al. 2006; López-Martínez et al. 2012, 2016). The EOZ-induced decrease in esterases may be interpreted as an inhibitory effect of  $O_3$  on the synthesis of  $\alpha$  and  $\beta$  esterases (Kurappasamy et 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  $\alpha$  and  $\beta$  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<sub>3</sub>-affected  
243 leaves or directly by breathing O<sub>3</sub> included in the air taken from the tracheae (a tubes  
244 network). This study aimed at investigative O<sub>3</sub> effects on the beetle as a sum of direct and  
245 indirect effects; insects would experience both direct and indirect effects in an O<sub>3</sub>-polluted  
246 environment. Hence, this study does not provide the opportunity to separate the direct and  
247 indirect effects of O<sub>3</sub> (Telesnicki 2015, 2018). However, previous bioassays, where this  
248 beetle was not directly exposed to O<sub>3</sub>, 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<sub>3</sub> on this beetle. The Japanese  
253 environmental quality standard for O<sub>3</sub> 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<sub>3</sub> 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<sub>3</sub> exposures  
258 lower than the standards. Rather, increased oxidative stress below the toxicological threshold

may promote health in animal models (Ristow and Schmeisser 2011). In fact, it is known that O<sub>3</sub> can induce the up-regulation of antioxidants in animal models, including humans, which are of an adaptive nature, resulting in potential effects that are beneficial to health (Bocci 2006, 2007, 2012). These effects may also protect against a subsequent more massive environmental threat, a phenomenon called *environmental conditioning* (Calabrese et al. 2016a,b; Agathokleous 2018). Adaptive responses should be further tested over time and across generations due to potential transgenerational hormetic mechanisms (Calabrese and Mattson 2017; Agathokleous 2018; Agathokleous et al. 2018).

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

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

O<sub>3</sub> 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  $\alpha$  and  $\beta$  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  $\beta$  esterases. ALP was negatively correlated with invertase. All other correlations were not significant.

#### 4. Conclusions

- EOZ increased the total antioxidant activity and decreased the  $\alpha$  and  $\beta$  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 ( $\alpha$  and  $\beta$ ) can be utilized as an effective O<sub>3</sub> biomarker system in this beetle.
- O<sub>3</sub>-induced biological responses of the beetle require further experimentations over time and across generations.

- A further challenging task would be to test whether potential adaptive responses of the beetle to sub-lethal O<sub>3</sub>-induced stress result in less harm following larger stress.

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Fig 1

**Fig. 1** The means ( $\pm$ SE) of total antioxidant activity (*A*), total carbohydrate content (*B*), and total protein content (*C*) in adult insects of *Agelastica coerulea* in ambient (AOZ) or elevated (EOZ) O<sub>3</sub> conditions. Data were statistically analyzed by the simple contrast AOZ vs. EOZ ( $n = 3$ ). *P*-values marked with bold indicate statistical significance at an  $\alpha$  level of 0.05.

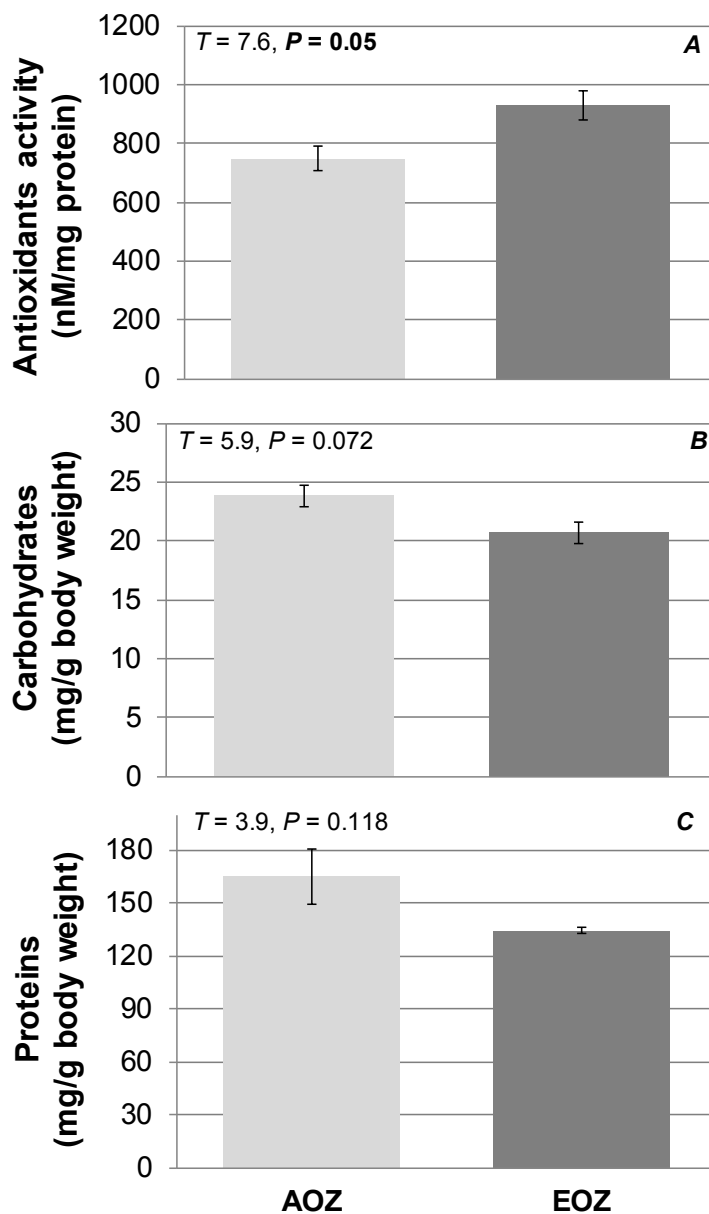


Fig 2

**Fig. 2** The means ( $\pm$ SE) of alpha (*A*) and beta (*B*) esterases in adult insects of *Agelastica coerulea* in ambient (AOZ) or elevated (EOZ) O<sub>3</sub> conditions. The units are ng  $\alpha$ -naphthol min<sup>-1</sup> mg<sup>-1</sup> protein for alpha esterases and ng  $\beta$ -naphthol min<sup>-1</sup> mg<sup>-1</sup> protein for beta esterases. Data were statistically analyzed by the simple contrast AOZ vs. EOZ ( $n = 3$ ). *P*-values marked with bold indicate statistical significance at an  $\alpha$  level of 0.05.

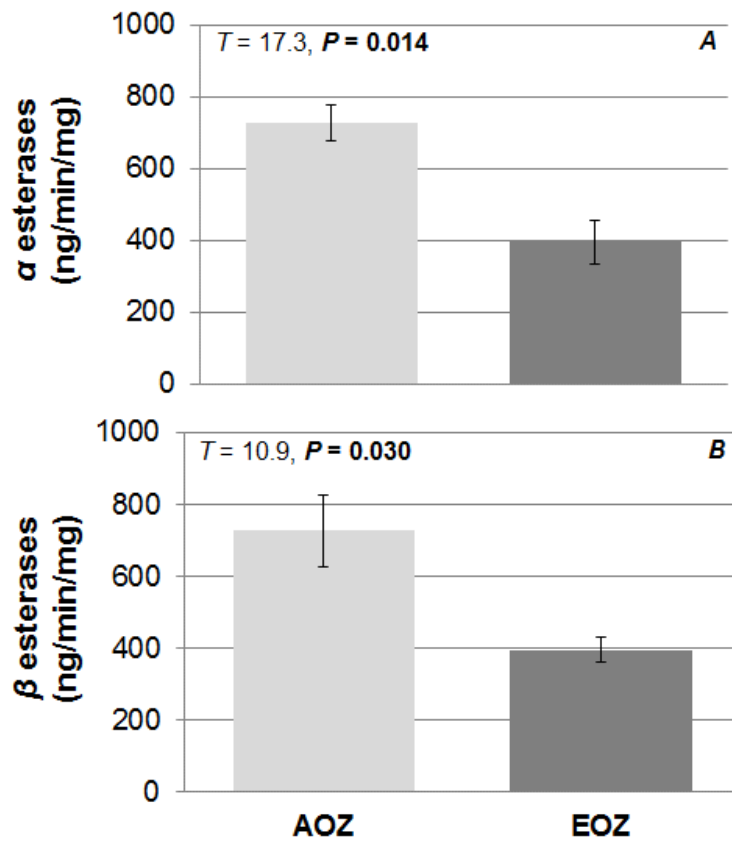


Fig 3

**Fig. 3** The means ( $\pm$ SE) of glutamic-oxaloacetic transaminase, GOT (*A*), glutamic--pyruvic transaminase, GPT (*B*), and lactate dehydrogenase, LDH (*C*) in adult insects of *Agelastica coerulea* in ambient (AOZ) or elevated (EOZ) O<sub>3</sub> conditions. Data were statistically analyzed by the simple contrast AOZ vs. EOZ ( $n = 3$ ), at an  $\alpha$  level of 0.05.

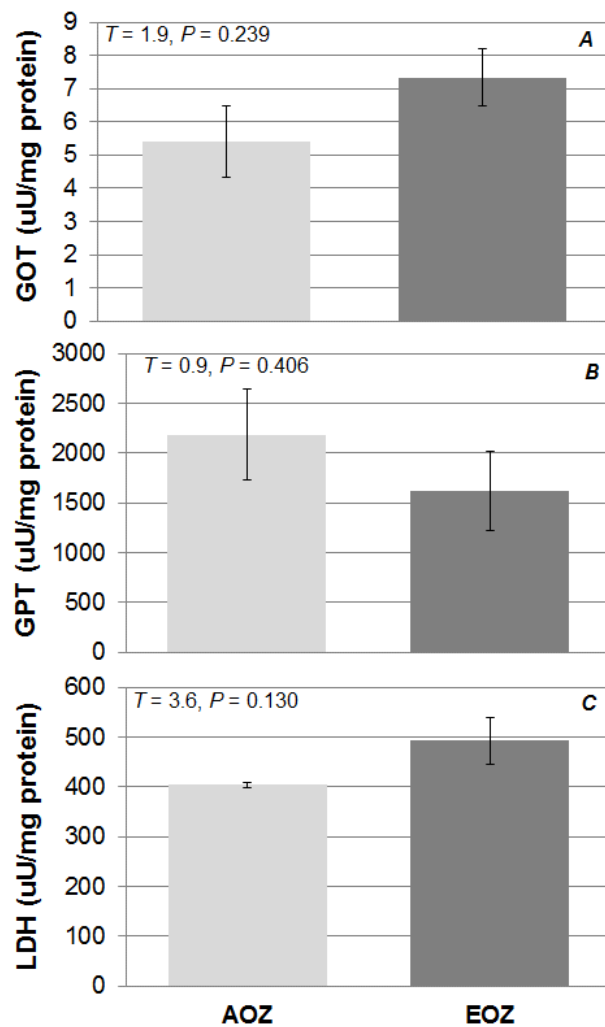


Fig 4

**Fig. 4** The means ( $\pm$ SE) of alkaline phosphatase, ALP (*A*), invertase (*B*), and trehalase (*C*) in adult insects of *Agelastica coerulea* in ambient (AOZ) or elevated (EOZ) O<sub>3</sub> conditions. Data were statistically analyzed by the simple contrast AOZ vs. EOZ ( $n = 3$ ), at an  $\alpha$  level of 0.05.

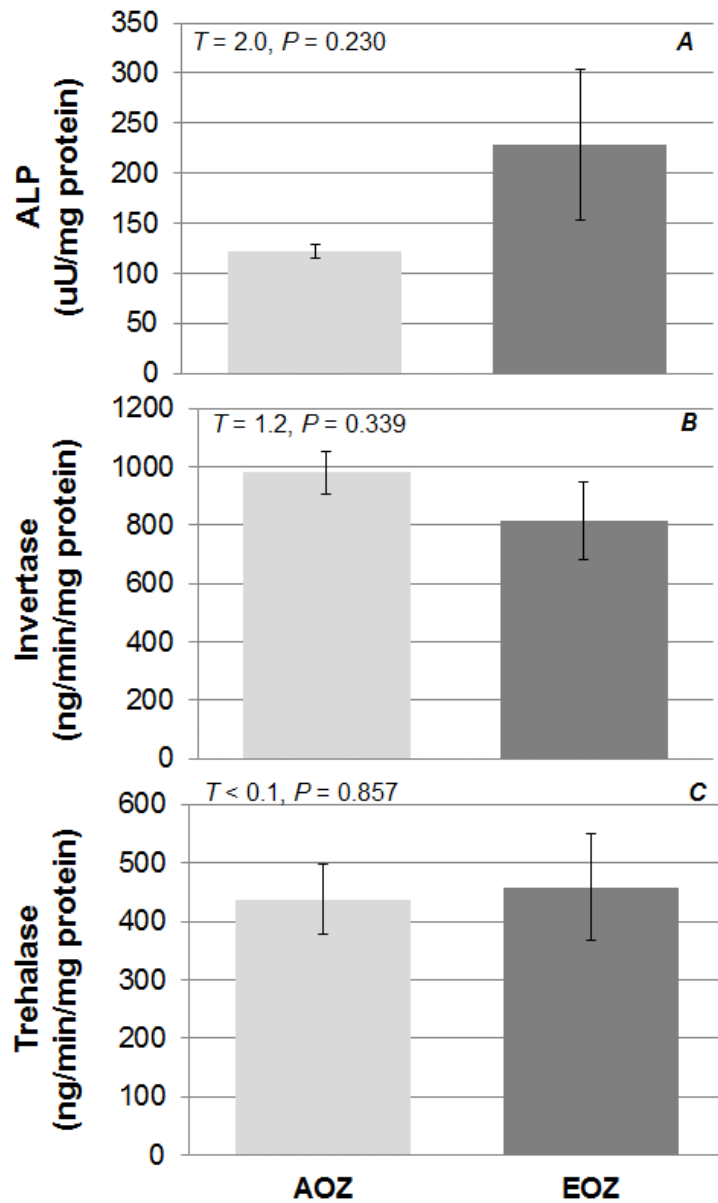


Fig 5

**Fig. 5** Biplot of the PCA ordination with varimax rotation based on T-scores of the response variables. Response variables on which elevated O<sub>3</sub> had a statistically significant effect are represented by black lines and markers, whereas those on which elevated O<sub>3</sub> had no statistically significant effect are represented by gray lines and markers. The response variables were: alpha esterases ( $\alpha$  esterases), alkaline phosphatase (ALP), beta esterases ( $\beta$  esterases), glutamic-oxaloacetic transaminase (GOT), glutamic--pyruvic transaminase (GPT), invertase, lactate dehydrogenase (LDH), total antioxidants, total carbohydrates, total proteins, and trehalase.

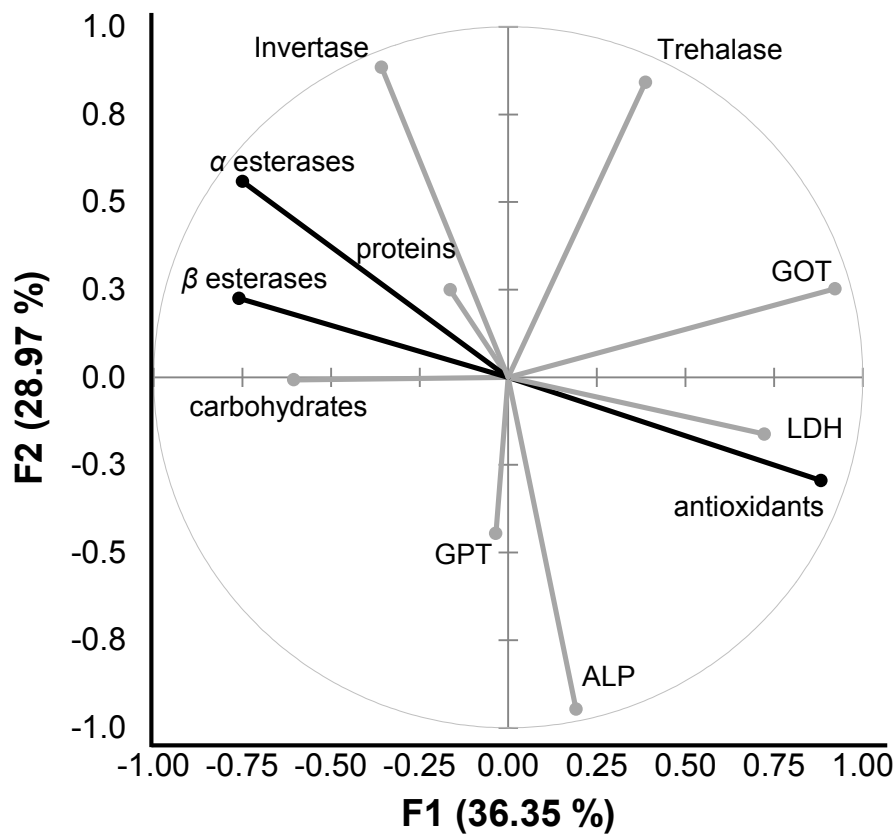


Table 1

**Table 1** The factor loading and the contribution of variable (%) after principal components analysis based on T-scores of the response variables. Response variables on which elevated O<sub>3</sub> had a statistically significant effect are represented by bold text, whereas those on which elevated O<sub>3</sub> had no statistically significant effect are represented by non-bold text. The response variables were: alpha esterases ( $\alpha$  esterases), alkaline phosphatase (ALP), beta esterases ( $\beta$  esterases), glutamic-oxaloacetic transaminase (GOT), glutamic--pyruvic transaminase (GPT), invertase, lactate dehydrogenase (LDH), total antioxidants, total carbohydrates, total proteins, and trehalase.

|                                      | Factor loadings |               |               | Contribution of the variables (%) |              |              |
|--------------------------------------|-----------------|---------------|---------------|-----------------------------------|--------------|--------------|
|                                      | F1              | F2            | F3            | F1                                | F2           | F3           |
| <b><math>\alpha</math> esterases</b> | <b>-0.750</b>   | <b>0.559</b>  | <b>0.178</b>  | <b>14.051</b>                     | <b>9.810</b> | <b>1.160</b> |
| ALP                                  | 0.191           | -0.947        | -0.129        | 0.916                             | 28.114       | 0.613        |
| <b><math>\beta</math> esterases</b>  | <b>-0.760</b>   | <b>0.225</b>  | <b>0.480</b>  | <b>14.443</b>                     | <b>1.594</b> | <b>8.452</b> |
| 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        |
| <b>Total antioxidants</b>            | <b>0.882</b>    | <b>-0.294</b> | <b>-0.285</b> | <b>19.449</b>                     | <b>2.718</b> | <b>2.984</b> |
| 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        |

559 Table 2

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

| Variables           | $\alpha$ esterases | ALP            | $\beta$ esterases | GOT     | GPT     | Invertase      | LDH           | Total antioxidants | Total carbohydrates | Total proteins | Trehalase |
|---------------------|--------------------|----------------|-------------------|---------|---------|----------------|---------------|--------------------|---------------------|----------------|-----------|
| $\alpha$ esterases  | 1                  | -0.7610        | 0.7205            | -0.5069 | 0.0155  | 0.7597         | -0.7144       | <b>-0.8284</b>     | 0.5266              | 0.4253         | 0.0818    |
| ALP                 | -0.7610            | 1              | -0.4021           | -0.0737 | 0.2396  | <b>-0.9138</b> | 0.3086        | 0.4384             | -0.1623             | -0.3953        | -0.6482   |
| $\beta$ esterases   | 0.7205             | -0.4021        | 1                 | -0.7387 | 0.2954  | 0.3904         | -0.5396       | <b>-0.8478</b>     | <b>0.8445</b>       | 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             | <b>-0.9138</b> | 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             | <b>0.8670</b>      | -0.7092             | -0.3927        | 0.1003    |
| Total antioxidants  | <b>-0.8284</b>     | 0.4384         | <b>-0.8478</b>    | 0.7040  | -0.0835 | -0.4648        | <b>0.8670</b> | 1                  | -0.7921             | -0.4680        | 0.0036    |
| Total carbohydrates | 0.5266             | -0.1623        | <b>0.8445</b>     | -0.5442 | 0.6135  | 0.0033         | -0.7092       | -0.7921            | 1                   | <b>0.8203</b>  | -0.1126   |
| Total proteins      | 0.4253             | -0.3953        | 0.6779            | -0.0954 | 0.6967  | 0.0848         | -0.3927       | -0.4680            | <b>0.8203</b>       | 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         |

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