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Plant oxidative status under ozone pollution as predictor for aphid population growth: The case of *Metopolophium dirhodum* (Hemiptera: Aphididae) in *Triticum aestivum* (Poales: Poaceae)

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ABSTRACT

Ozone is a secondary air pollutant that affects plants and animals through several physiological mechanisms that involve changes in redox status. However, the consequences of ozone pollution on aphids are not well understood. Therefore, we have experimentally tested: if oxidative stress on the host plant affects lipid peroxidation in aphids or aphid population growth. Wheat plants (*Triticum aestivum*) were exposed to 140 p.p.b. of ozone or filtered air in open top chambers for three consecutive days and *Metopolophium dirhodum* (Walker, 1849, Hemiptera: Aphididae) aphids were transferred to the plants immediately after ozone exposure or 72 h later. Ozone exposure reduced antioxidant potential within plant tissues and had no effect on plants' lipid peroxidation. Lipid peroxidation in aphids fed upon these plants was similar among treatments. Although aphids successfully colonised the plants in all the treatments, the populations established on plants immediately after ozone exposure grew at higher rates than those established 72 h after ozone exposure had ended, independently of ozone level. In conclusion, aphids were tolerant to plant mediated effects of ozone. Therefore, a greater attention should be put in the direct effects of ozone on *M. dirhodum* - *T. aestivum* interaction.

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1. Introduction

Ozone has an important biological impact on plants and animals, besides its effect as a greenhouse gas (Myhre et al., 2013). Ozone is largely produced in the lower atmosphere from primary air pollutants, such as nitric oxides, sulphur oxides, carbon oxides and hydrocarbons in the presence of sunlight (Iriti and Faoro, 2009). As any photochemical pollutant, ozone formation depends on solar radiation. This leads to its episodic and cyclic nature (Booker et al., 2009; Schnell et al., 2009; Vingarzan, 2004). Besides the increase in the background concentrations of ozone during the past century (Vingarzan, 2004), acute ozone episodes that reach over 120 ppb during the day currently occur at diverse locations (Assareh et al., 2016; Domínguez-López et al., 2015; Schnell et al., 2009) and have a negative impact on vegetation and food production (Avnery et al., 2011).

This negative effect of ozone arises from the disturbance of the equilibrium between production and scavenging of reactive oxygen species (ROS), within animal and plant tissues (Iriti and Faoro, 2007). The outermost biological surfaces have an antioxidant system which provides a primary defence against atmospheric ROS (Cross et al., 2002). When this barrier is overcame, ROS enter the cells and produce an oxidative burst which is counteracted by a diverse set of soluble (ascorbate, glutathione, tocopherol, carotenoids and phenolic compounds) and enzymatic antioxidants (superoxide dismutase, catalase, glutathione peroxidase, guaiacol peroxidase, peroxiredoxins and enzymes of the ascorbate-glutathione cycle) (Caverzan et al., 2016; Fangmeier et al., 1994; Foyer and Noctor, 2005; Li et al., 2013; Valkama et al., 2007; Wang et al., 2014). As ozone enters plant cells, it produces ROS such as H_2O_2, superoxide (O_2^-) and hydroperoxyl (HOO^-) radicals (Ahsan et al., 2010). The following oxidative burst involves changes in the oxidative signalling pathways through the production of ROS (Baier et al., 2005; Foyer and Noctor, 2005; Kangasjärvi et al., 2005) and upregulates the expression of proteins associated with antioxidant defense mechanisms, carbon metabolism, secondary

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metabolism and nitrogen metabolism (Ahsan et al., 2010). Moreover, ozone downregulates the expression of proteins associated with photosynthesis pathways (Ahsan et al., 2010), ultimately reducing carbon uptake, and/or photosynthetic carbon fixation, with consequences on plant growth and on the translocation of fixed carbon to other plant tissues (Wilkinson et al., 2012).

Several parallelisms can be established between plants and animals in terms of their susceptibility to ozone injury, as antioxidant defences have been highly conserved along evolutionary history. For instance, plants' hypersensitive response (HR) is frequently compared to animal inflammatory responses (Cross et al., 2002). Insects are susceptible to oxidative stress (Cross et al., 2002; Holmstrup et al., 2011; Telesnicki et al., 2015) and to the accumulation of ROS (Smith and Boyko, 2007). In the case of aphids, antioxidants play an important role in terms of nutrition, defence against environmental stress and coping with ROS mediated plant defence (Goggin et al., 2010; Kerchev et al., 2012; Mai et al., 2013). Aphids have a complex feeding behaviour, which allows them to furtively feed on plant tissues without causing major injuries (Züst and Agrawal, 2016). Additionally, the salivary secretions of aphids modulate or suppress the phytohormonal and defensive response of susceptible plants and modify source-sink relationships in the translocation of nutrients (Giordanengo et al., 2010; Goggin, 2007; Powell et al., 2006; Züst and Agrawal, 2016).

Aphid-plant interactions under ozone pollution are not clearly understood. Under ozone stress, individual and population growth rates, developmental time and fecundity of aphids either increase, decrease or remain untouched (Awmack et al., 2004; Brown et al., 1992; Holopainen, 2002; Holopainen and Kossi, 1998; Jackson, 1995; Menéndez et al., 2010; Mondor et al., 2010; Warrington, 1989). Ozone may affect aphids directly (Telesnicki et al., 2015), indirectly or by the interaction of direct and indirect effects, when plants and aphids are simultaneously exposed to ozone (Awmack et al., 2004; Brown et al., 1992; Holopainen and Kossi, 1998; Menéndez et al., 2010; Mondor et al., 2010; Warrington, 1989). Simultaneous exposure of plant and aphids to ozone offers a realistic approach to study ozone's effect on aphid-plant interaction. However, the isolated exploration of the direct and indirect effects of ozone on aphids allows a clearer distinction of ozone's effect on aphids from the sum of effects of ozone on each member of this interaction. In the case of the direct exposure of aphids to ozone, ozone has been shown to lead to oxidative stress accumulation, increased mortality and reduced aphids' dispersion ability (Telesnicki et al., 2015).

Regarding the indirect effects of ozone on aphids, two main mechanisms have been considered to explain ozone-driven changes in aphid populations: (1) changes in plant nutritional quality (reviewed in Valkama et al., 2007; Dermody et al., 2008) and (2) the activation of plants crossed-response to biotic and abiotic stress factors (crosstalk) through modification of the oxidative status of the plant (Menéndez et al., 2009). On one hand, no correlation was found between nutrient content and aphid performance in increased ozone environments (Dermody et al., 2008; Valkama et al., 2007). Actually, in these studies, ozone had no consistent effect on either carbon (C) concentration, nitrogen (N) concentration, C:N ratio, or on the relative growth rate of individual aphids (RGR) or population size (Dermody et al., 2008; Valkama et al., 2007). On the other hand, several secondary metabolites with antioxidant capacity, such as phenolic acids, flavonoids, glutathione and ascorbate have been shown to increase significantly after plant exposure to ozone (Fangmeier et al., 1994; Foyer and Noctor, 2005; Valkama et al., 2007; Wang et al., 2014). Moreover, aphids can benefit from feeding on plants with enhanced antioxidant content (Kerchev et al., 2012). As abiotic stress also leads to antioxidant accumulation (Kangasjärvi et al., 2005; Sharma and Davis, 1997), it has been hypothesized that it could reduce the effectiveness of plant defence against insects (Łukasik and Goławska, 2013).

Therefore, the aim of this study is to evaluate the indirect effect of ozone on aphids at biochemical scale and its impacts at aphid population scale. We conducted two independent experiments to test the following hypothesis: 1) oxidative stress accumulation in aphids depends on plants oxidative stress status and 2) ozone-induced increase in plant antioxidant potential has a positive effect on aphid population growth.

2. Materials and methods

Two independent experiments were conducted at IFEVA (Faculty of Agronomy, University of Buenos Aires, 34° 35′S, 58° 29′W) to test the abovementioned hypotheses. Treatments were designed to mimic the occurrence of acute ozone episodes and patchy aphid infestations occurring before or after the plant exposure to the contaminant. The aphid oxidative stress experiment was aimed at testing the first hypothesis and the aphid population growth experiment was aimed at testing the second hypothesis. In both experiments, the plants were exposed to ozone or charcoal filtered air for three consecutive days before receiving the aphids. Aphids were not exposed to ozone at any moment, as the experiments were designed to exclusively evaluate the indirect effect of ozone on aphids. Since ozone-induced changes in plant antioxidant potential vary over time (Kangasjärvi et al., 2005), the indirect effect of ozone was evaluated at two different moments: 0 h after ozone exposure and 72 h after exposure had ended.

2.1. Plants

A total of 140 spring wheat plants (*Triticum aestivum* L. cv. 'Cronox', Don Mario, Chacabuco, Argentina) were individually grown in 2 L plastic pots containing a 50% soil, 25% peat moss and 25% perlite potting mixture and were used for both experiments. The pots were placed inside plastic containers with a water reservoir to keep the soil under constant moisture. Plants were kept in a glasshouse (mean temperature 18.5 °C) until tillers were completely formed. Then, they were transferred to the open top chambers to allow for plant acclimation one week prior to ozone exposure.

2.2. Ozone exposure

Plant exposure to ozone was performed in 8 m^3 "open-top" chambers (OTC) with crystal PVC (polyvinyl chloride) walls mounted on a metal structure which allowed ozone level regulation (Hogsett and Tingey, 1985; Lefohn et al., 1986). Ozone was generated from charcoal-filtered air by a spark discharge-type ozone generator (Dobzono, Buenos Aires, Argentina). Ozone concentration inside the OTC was continuously monitored using a Model 450 Ozone Monitor API-Teledyne Instrument (Teledyne Advanced Pollution Instrumentation, San Diego, CA). The eight chambers were laid in a radial array and ozone level was randomly assigned to each chamber. Each OTC was provided with an air conditioning system. Mean (±SEM) temperature within the OTC during ozone exposure was 24.6 °C ± 1.1 °C. Near surface ozone currently reaches maximum concentrations over 100 p.p.b. (Andersson et al., 2017; Wang et al., 2017) and projections also show increases in background ozone levels (Lin et al., 2017; Sicard et al., 2017). Therefore, ozone and filtered air were mixed in different proportions to obtain two contrasting ozone exposure conditions: 0.0 ± 0.7 p.p.b. or 140 ± 14 p.p.b. The plants received an acute, 5-h ozone exposure treatment during three consecutive days, which is sufficient to induce changes in antioxidant related gene expression and antioxidant enzymatic

activity changes in wheat (Li et al., 2013). Afterwards, none of the plants received ozone.

2.3. Aphids rearing

Metopolophium dirhodum (Walker, 1849, Hemiptera: Aphididae) specimens were collected from spontaneous populations growing on *Lolium multiflorum* (Lam.) and other Gramineae at IFEVA, Facultad de Agronomía, Universidad de Buenos Aires experimental field. These field-collected aphids were initially kept on individual leaves to discard parasitized individuals. Unparasitized aphids were reared in wheat plants (*Triticum aestivum* L. cv Cronox) under controlled temperature (22 °C) and photoperiod (L12: D12). One month later, apterous adults and large immature aphids were selected for the experiments.

2.4. Aphids oxidative stress experiment

Aphids were allowed to feed on control and ozone treated plants immediately or 72 h after ozone exposure for 48 h. During this 48 h feeding period, plants were kept inside the OTC in which ozone exposure had taken place. After the plant exposure period, none of the OTC received ozone, but charcoal filtered air only.

Each determination of the oxidative stress biomarkers on aphids required 100 individuals. Then, twenty-five to thirty apterous aphids of homogeneous size were individually transferred (with a fine brush) to the leaves of a single plant and were later pooled to recover enough material for each determination. Each plant was surrounded by a 100 μm translucid PET cylinder from the base of the pot to 10 cm above the leaves to keep aphids confined.

A total of eighty wheat plants were used for this experiment, divided among 8 OTC (four received air mixed with ozone and the remaining received charcoal filtered air). From the ten plants assigned to each chamber, eight were divided between the two time levels (0 or 72 h after ozone exposure) and received aphids. The remaining two plants from each chamber received no aphids and were destructively sampled for oxidative stress determinations (lipid peroxidation and antioxidant potential, characterized below) at each time. Aphids were placed on leaves at the same position as those from which the biochemical determinations had been done in homologous plants, independently of the presence of visible injury on the leaf and as long leaves were not completely damaged. Each treatment combination (ozone exposure x time after exposure) had four replicates.

Lipid peroxidation and antioxidant potential were also assessed in wheat leaves from an additional set of 12 plants that had been exposed to ozone or charcoal filtered air simultaneously with the previously mentioned plants. That is, each of three OTC from the ozone treatment and each of three OTC from the filtered air treatment received two additional plants which were destructively sampled at the end of the first and second day of ozone exposure (three replicates of each ozone level, each day). The end of the third day of ozone exposure coincided with the 0 h time level (four replicates at each ozone level).

2.5. Aphid population growth experiment

Plants and aphids used for this experiment were grown and exposed to ozone as explained above, following the same ozone x time after exposure treatments and criteria for aphid selection. In contrast with the previous experiment, 48 plants were divided among a total of six OTC (three received ozone mixed with filtered air and three received filtered air). Within each chamber, the eight plants were divided between the time levels (0 or 72 h level). Two of these four plants were used for oxidative stress determinations and two received 10 aphids each. The number of successfully established aphids was determined as the number of surviving aphids feeding or walking on the plant 24 h later. The aphids were recounted 1, 3, 5, 10 and 15 days after being placed on the plants to estimate population growth. As in the aphids oxidative stress experiment, the plants were surrounded by a translucid PET cylinder, but afterwards kept inside the glasshouse (mean temperature 18.51 °C). Each ozone x time combination treatment was replicated six times.

2.6. Oxidative stress biomarkers

Lipid peroxidation was assessed by thiobarbituric acid reactive species technique (TBARS) following a protocol modified after Lissi et al. (1995) and Łukasik et al. (2009). Aphid and plant samples were collected, immediately frozen in liquid nitrogen and stored at −80 °C until the biochemical determinations were performed. Each plant sample consisted of three or four fully expanded wheat leaves from the same position at different tillers of the same plant to reach 1 g of plant leaf material. If any leaf showed extensive injury it was replaced by homologous leaves from different tillers to keep samples' homogeneity. Plant samples were homogenized in 1% phosphoric acid at 0 °C for 5 min. The homogenates were filtered through two layers of cheesecloth and centrifuged at 3000 g for 15 min. The pellets were discarded and the supernatants were used in the reaction mixture. In the case of aphid samples, aphid tissue homogenates were obtained from 100 individual batches homogenized with phosphate buffer (30 mM, KCl 120 mM, pH = 7.4) in place of phosphoric acid. The reaction mixture consisted of aphid or plant tissue homogenate, 4% BHT in ethanol, 20% TCA and 0.7% TBA. After 1 h of incubation at 100 °C, the MDA-TBA complex yielded by this reaction was spectrophotometrically quantified at 532 nm ($\varepsilon = 1.56\ mM^{-1}\ cm^{-1}$). Total reactive antioxidant potential (TRAP) was assessed only in plants (Lissi et al., 1995), while TBARS were assessed in plants and aphids.

2.7. Statistical analysis

Both experiments were designed following a split-plot design. The main plot corresponded to the OTC and the sub-plot corresponded to the time after exposure treatment. All the variables were analyzed using a hierarchical linear model with ozone exposure and time after exposure as fixed effects and OTC as random effect. Analyses were performed with linear mixed effect models with the package nlme (Pinheiro et al., 2015) using statistical software R (R-cran Project). The statistical model always followed the experimental design and model selection was not applied in any case. As different variables were considered, the following distinctions apply: the protein content of the tissue extract was included as covariate in the models regarding TBARS and TRAP and a normal distribution was assumed for these variables. In the aphid population growth experiment, aphid population growth curves were calculated with the repeated measurements of each plant, and the intrinsic rate of aphid population increase was calculated as the slope of the log-linearized regression model equations. A normal distribution was also assumed in the case of the intrinsic rate of population increase and a binomial distribution was assumed for aphid colonisation success. The distribution of the residuals was checked in all cases.

3. Results

3.1. Aphids oxidative stress experiment

Plants exposed to ozone showed chlorotic and necrotic

symptoms, while plants exposed to filtered air showed no signs of injury. Ozone exposure diminished plants' TRAP irrespectively of time level (Fig. 1A, $p = 0.03$). In addition, the contents of TRAP and TBARS (Fig. 1A and B) were higher in plants from the 0 h treatment than in those from the 72 h treatment (TRAP: $p = 0.01$, TBARS: $p = 0.02$). Additionally, the negative effect of ozone on plants' TRAP was also registered along the exposure period (Fig. 2A). TRAP decreased with ozone ($p = 0.005$) and day of exposure (day 2: $p = 0.000$, day 3: $p = 0.0001$). However during ozone exposure, ozone-time interaction alleviated the negative effects of the isolated factors (ozone-day 2: $p = 0.006$). Despite of ozone's and time effect on TRAP, they had no effect on plants' lipid peroxidation (TBARS, Fig. 2B, $p = 0.068$). Lipid peroxidation in aphids (TBARS, Fig. 1C) was not affected by plants' previous exposure to ozone ($p = 0.38$) or the time elapsed after ozone exposure ($p = 0.82$).

3.2. Aphid population growth experiment

Unaffected by ozone exposure ($p = 0.45$, Fig. 3A), aphid population growth rate was higher for aphids transferred to plants immediately after ozone exposure than for those transferred to plants 72 h after ozone exposure($p = 0.04$, Fig. 3A). However, aphid colonisation success (Fig. 3B) was not affected by ozone treatment ($p = 0.84$) or time ($p = 0.82$). Although plants exposed to ozone showed visible chlorotic and necrotic symptoms, plants' TBARS and

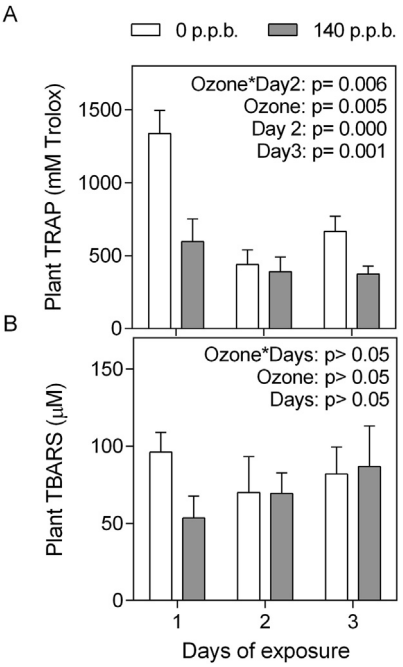

Fig. 2. Plants response to ozone during exposure. (A) Mean (±SEM) antioxidant potential (TRAP) and (B) lipid peroxidation (TBARS) at the end of each of three day ozone exposure ($n = 3$ for days 1 and 2, $n = 4$ for day 3).

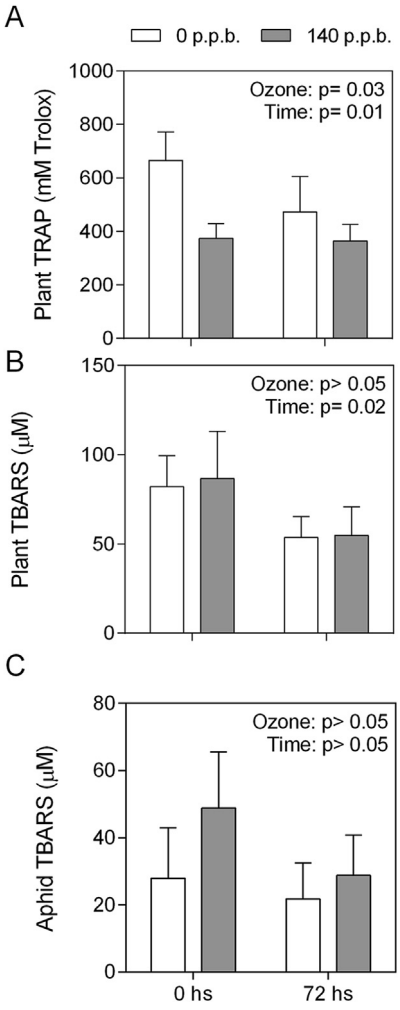

Fig. 1. Aphids oxidative stress experiment. Clear bars correspond to 0 p.p.b. ozone and grey bars correspond to 140 p.p.b. ozone. Mean (±SEM) (A) antioxidant potential (TRAP) in plants at the beginning of aphid feeding ($n = 4$). (B) Lipid peroxidation (TBARS) in plants and (C) lipid peroxidation (TBARS) in aphids after 48 h of feeding on plants that had just ended ozone exposure (0 h) or that had ended ozone exposure 72 h before ($n = 4$).

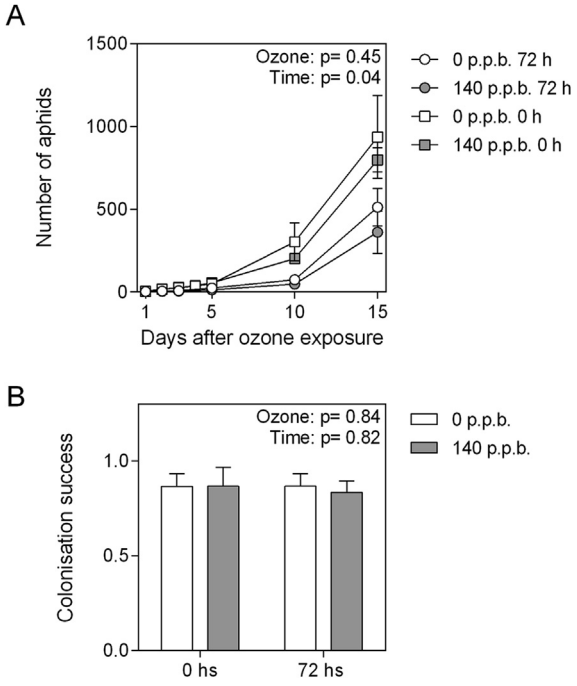

Fig. 3. Aphid population growth experiment. (A) Mean (±SEM) number of aphids along time (clear icons = 0 p.p.b. ozone, black icons = 140 p.p.b. ozone, square icons = 0 h, round icons = 72 h after ozone exposure, $n = 6$). (B) Mean (±SEM) colonisation success of aphids on 0 p.p.b. ozone (clear bars) or 140 p.p.b. ozone (grey bars) exposed plants and offered to aphids immediately after ozone exposure (0 h) or 72 h after ozone exposure ($n = 6$).

TRAP (Table 1) were not affected by ozone exposure ($p = 0.45$ and $p = 0.61$, respectively) or time ($p = 0.39$ and $p = 0.89$, respectively).

4. Discussion

This study demonstrates that *M. dirhodum* aphids are tolerant to plant mediated (i.e. indirect) effects of ozone at biochemical and population scale, despite ozone induced changes in wheat plants. Ozone induces an oxidative burst in plants, which is later followed by induction of ROS- scavenging soluble antioxidants and enzymes with antioxidant activity, that counteract the toxic effects of high ROS concentration (Apel and Hirt, 2004; Bender et al., 1994; Li et al., 2013). The oxidative burst was evidenced by visible necrotic symptoms and the decrease of TRAP during ozone exposure (Fig. 2A). However, lipid peroxidation in aphids and insect population growth were not affected by prior exposure to ozone on plants. As TRAP is mainly associated to the soluble antioxidants (as carotenoids and tocopherol) it remains possible that ozone induced changes in enzymes with antioxidant activity like superoxide dismutase, peroxidase (Li et al., 2013) which were not considered in this study, could compensate for ozone driven oxidative stress and leave aphids unaffected. As the effect of abiotic stress is genotype-specific (Caverzan et al., 2016), a more detailed study of antioxidant enzymes expression and activities under ozone pollution would be of interest in order to elucidate the mechanisms behind the observed pattern. Superoxide dismutase, catalase, ascorbate peroxidase, guaiacol peroxidase and glutathione reductase have been identified as antioxidant enzymes susceptible to changes under several abiotic stress conditions in wheat (Bender et al., 1994; Caverzan et al., 2016; Li et al., 2013) and should be considered in future studies regarding ozone effect on wheat plants.

As aphid - plant interactions are diverse in terms of plant defence mechanisms and aphids strategies to overcome these defences which also involve ROS and antioxidants (Giordanengo et al., 2010; Goggin, 2007; Züst and Agrawal, 2016), it becomes difficult to predict the result of these interactions under a scenario of high ozone pollution without isolating the direct and the indirect effects of ozone at different scales and taking in consideration the characteristics of the interacting aphid and plant species. Under ozone pollution, the population growth rate of some aphid species depends on the oxidative status of both plants and aphids (Menéndez et al., 2013). When arugula plants were exposed to ozone, their antioxidant potential was increased and *Myzus persicae* later feeding on them experienced a higher population growth rate and reached higher densities than on control plants (Menéndez et al., 2013).However, in our experiment, ozone exposure did not increase plants' antioxidant potential and aphid population growth remained unaffected by ozone. Additionally, *M. dirhodum* intrinsic population growth rates ranged within values expected for unstressed populations (Fereres et al., 1989; Harmon et al., 2009).

Despite being generally accepted that ozone effects on aphids are mainly indirect (Holopainen, 2002), results from our previous work (Telesnicki et al., 2015) and the present study highlight the value of conducting separate experiments to assess the relative importance of direct and indirect effects of ozone on aphids. *M. dirhodum* is susceptible to oxidative damage, reduced mobility and a greater mortality due to direct effect of ozone (Telesnicki et al., 2015), while no indirect effect of ozone was registered in the current experiments. Therefore, in the case of *M. dirhodum* – *T. aestivum* interaction, a greater attention should be paid to the direct effects of ozone.

In sum, the relative importance of the direct and indirect effects of ozone on aphids varies among the pairs of species under consideration and future studies that assess the isolated effects of ozone on the interaction of different aphid and plant species will be of importance to predict the possibility of aphid outbreaks in polluted environments. In the case of *M. dirhodum*, population outbreaks increase the risk of aphid-transmitted virus epidemics, such as Barley Yellow Darf Virus (BYDV). As the changing levels of atmospheric gases could modify the effects of diseases on organisms or ecosystems, it is also important to study if virus derived benefits on aphids, as increased free amino-acid concentration in plant sap (Fereres et al., 1989), and/or the virus mediated manipulation of aphid behaviour (Fereres and Moreno, 2009; Ingwell et al., 2012) persist under ozone pollution.

Table 1
Mean (±SEM) lipid peroxidation (TBARS, μM) and total reactive antioxidant potential (mM Trolox) of wheat plants which had been previously exposed to ozone (140 p.p.b.) or charcoal filtered air (0. p.p.b.) and offered to the aphids immediately after ozone exposure (0 h) or 72 h after the end of exposure at aphid population growth experiment ($n = 6$).

Ozone	0 p.p.b.		140 p.p.b.	
Time	0 h	72 h	0 h	72 h
Plants TBARS	11.95 ± 1.66	13.58 ± 1.03	10.30 ± 1.02	9.16 ± 1.14
Plants TRAP	251 ± 49	219 ± 23	249 ± 53	310 ± 38

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