Mutualism effectiveness of a fungal endophyte in an annual grass is impaired by ozone

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Summary

1. Ozone is an increasing tropospheric contaminant of climate change. Exposure to ozone may affect the symbiotic relationship between plants and beneficial microorganisms. In particular, the herbivore resistance mechanism conferred by fungal endophytes (defensive mutualism) may be affected, as any of the ozone-triggered effects (such as elicitation of defence mechanisms against biotrophic fungi or oxidative stress in the apoplastic space) may target the symbiont.
2. Symbiotic and non-symbiotic *Lolium multiflorum* plants were exposed to ozone for two consecutive days (2 h per day), after which half were infested with 10 aphids (*Rhopalosiphum padi*). We measured variables related to performance of plants, the endophyte symbiont (alkaloids) and the herbivores.
3. Aphid populations were smaller on symbiotic plants than in non-symbiotic plants in low-ozone conditions. However, this difference disappeared in exposed plants to high-ozone conditions. Under low-ozone conditions, structure of aphid populations on endophyte-symbiotic plants was characterized by a low number of nymphs and a high number of adults. This pattern was not observed with high-ozone exposure. Level of fungal alkaloids (lolines) was not affected by either ozone or herbivory.
4. Results indicate that ozone impairs the grass-endophyte symbiosis without affecting production of alkaloids generally linked with aphids' response to endophyte presence. Since neither plant biomass nor alkaloid level were affected by ozone, other ozone-mediated mechanisms at molecular or biochemical level may underlie plant-herbivore interaction mediated by fungal endophytes. Thus, the mechanism behind this effect must be determined in future experiments.

Key-words: alkaloids, aphids, endophyte, grass-endophyte interaction, *Lolium multiflorum*, ozone, *Rhopalosiphum padi*, symbiosis

Introduction

Research activity has greatly increased in the last few years examining effects on the environment, community structure and functioning of ecosystems in light of anthropogenic climate change (Lindroth 2010; Saikkonen *et al.* 2012). One aspect focuses on understanding the impact of factors causing oxidative stress such as UVb and ozone, on the plant interactions with other species in the same or different trophic level (Booker *et al.* 2009; Kiers *et al.* 2010; Lindroth 2010; Menéndez *et al.* 2010; Ballaré *et al.* 2012; Landesmann *et al.* 2013; Pineda *et al.* 2013). Ozone, in particular, is recognized as a tropospheric pollutant that causes oxidative stress in organisms, and it is known to affect plant-to-plant communication (Blande, Holopainen & Li 2010) and the interaction of plants with herbivores and microorganisms (Kytöviita, Le Thiec & Dizengremel 2001). Alternatively, ozone can be an elicitor of plant hormonal pathways such that it can induce defences against herbivores and pathogens (Kangasjärvi *et al.* 1994; Sandermann *et al.* 1998; Kangasjärvi, Jaspers & Kollist 2005). These plant-mediated effects of ozone on herbivores' performance have not been explored when the defence is conferred by symbiont microorganisms.

Ozone is a leading component of photochemical air pollution, in part, because of the photochemical reactions of gases resulting from human activities (Agrell *et al.* 2005; IPCC 2007; Booker *et al.* 2009). It enters plants through the stomata and diffuses by the apoplast, where reactive oxygen species (ROS) and free radicals of high oxidative

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power are produced as a consequence of its interaction with organic molecules and cellular components (Kangasjärvi, Jaspers & Kollist 2005; Tamaoki 2008). The ROS are part of the basal metabolism of the plant, and are involved in signalling pathways at the cellular level. However, ozone can significantly increase the level of ROS production and depending on the intensity and duration of events, it may also cause apoptosis (programmed cell death), tissue damage and organism's death (Kanofsky & Sima 1995; Kangasjärvi, Jaspers & Kollist 2005). In cultivated plants, its negative effects can scale up from photosynthesis and growth, to final yield (Agrell *et al.* 2005; Fiscus, Booker & Burkey 2005; Martínez-Ghersa, Olszyk & Radosevich 2008; Tamaoki 2008; Booker *et al.* 2009). An antioxidant network – where several secondary compounds and enzymes are involved – prevents the ROS from reaching damaging levels (Mittler 2002; Kranner *et al.* 2010). Even though the antioxidant-mediated defence system can be stimulated by a stress factor, it can be overcome leading to the aforementioned negative effects (Tamaoki 2008; Menéndez *et al.* 2009). The gaseous nature of ozone makes its incidence highly episodic, though seasonal and daily patterns matching the solar radiation dynamics are detectable (Booker *et al.* 2009; Schnell *et al.* 2009). With a mean environmental level of around 40–50 ppb (parts-per-billion), daily ozone can peak around 120–140 ppb, which is more than enough to trigger the negative effects previously described (Tamaoki 2008; Schnell *et al.* 2009). In highly contaminated areas several days of ambient smog exposure, involving 1-h ozone concentration peaks in the range of 120–190 ppb, might occur throughout the growing season (Booker *et al.* 2009).

Herbivory is a major threat to plants and the occurrence of insect outbreaks are expected to increase under global climate change scenarios (Williams & Liebhold 1995; Coviella & Trumble 1999; De Sassi & Tylianakis 2012; Jamieson *et al.* 2012). Plants, however, contain a broad arsenal of mechanisms to diminish the impact of herbivory (so-called defences) and to affect negatively the performance of the enemy (so-called resistance) (Karban & Baldwin 1997; Agrawal 2000; Ballaré *et al.* 2012; Thaler, Humphrey & Whiteman 2012). As a result, efforts have been made on predicting the consequences of environmental variables such as temperature, precipitation and CO_2, on different aspects of plant-herbivore interaction. Reaction with CO_2 (see Bezemer, Jones & Knight 1998; Bezemer *et al.* 1999; Newman *et al.* 1999, 2003; Whittaker 1999, 2001; Hughes & Bazzaz 2001; Ryan *et al.* 2014) and effects of ozone on the interactions between plants and aphids have been unclear and highly idiosyncratic (Holopainen 2002; Valkama, Koricheva & Oksanen 2007; Lindroth 2010). Effects of ozone on herbivore performance may be direct by affecting their physiology, or indirect through phytochemical changes of exposed plants (Lindroth 2010). The exposure magnitude in terms of intensity and duration will determine the plant response to acute or chronic stress by ozone. Episodic exposures may

be associated with accumulation of secondary compounds such as phenolics (e.g. flavonoids), terpenoids and antioxidants (Valkama, Koricheva & Oksanen 2007; Lindroth 2010) and particularly, with the elicitation of the salicylic acid defence signalling pathways (Sandermann *et al.* 1998; Menéndez *et al.* 2009). Interestingly, cross-talk between different defence mechanisms at the plant biochemical level (Karban & Baldwin 1997; Thaler, Humphrey & Whiteman 2012) may generate convergence between the effects of ozone and certain action from the herbivores, so that one agent may diminish the impact of the second (Sandermann *et al.* 1998; Menéndez *et al.* 2009, 2010). For example, sap-sucking herbivores such as aphids were found to reduce the negative impact of ozone in plants of arugula (*Eruca sativa* Mill), although ozone also affected aphids adversely (Menéndez *et al.* 2010). It is unclear however, whether the episodic exposure of plants to ozone affects the aphid performance indirectly through the plant.

Besides this complex system of defences, plants have found other ways to defend themselves through the association with beneficial microorganisms (Clay 1988; Karban & Baldwin 1997; Saikkonen, Gundel & Helander 2013). It has been proposed that the mutualism between cool-season grasses (subfamily Pooideae) and vertically transmitted epichloae fungal endophytes (Ascomycota, Clavicipitaceae) have evolved from a pathogenic relationships, to a state where plants have gained resistance to herbivory (Bush, Wilkinson & Schardl 1997; Clay & Schardl 2002; Selosse & Schardl 2007; Schardl 2010). In particular, loline alkaloids produced by several epichloae species are potent, broad-spectrum insecticides, which are generally linked to endophyte bioprotective effects against aphids (Wilkinson *et al.* 2000; De Sassi, Müller & Krauss 2006; Schardl 2010). Apart from the alkaloids mediating the resistance to herbivory, the symbiosis is associated with an increased tolerance to abiotic stress in general (Malinowski & Belesky 2000; Clay & Schardl 2002). Recently, an increased load of antioxidants in symbiotic plants has been proposed to be responsible for the higher tolerance to abiotic stress (White & Torres 2010; Hamilton *et al.* 2012). A fine-tuned balance between ROS and antioxidants seems to determine the interaction stability among grasses and endophytes (Tanaka *et al.* 2006; Hamilton *et al.* 2012). However, the ecological stability of the mutualism in terms of the effectiveness of the anti-herbivory mechanism under stress by ozone exposure has not been explored.

The interaction between these two strategies of plant defences, of the plant itself and the one conferred by fungal endophytes, has been scarcely studied (Simons, Bultman & Sullivan 2008; Saikkonen, Gundel & Helander 2013). Here, we use the framework of hormonal plant defence mechanisms in interaction with the one conferred by epichloid fungal endophytes, to explore the consequences of ozone on the resistance to sap-sucking herbivores in grasses. Based on antecedents showing that endophytes usually increase plant tolerance to abiotic stress (Malinowski & Belesky 2000; White & Torres 2010;

Hamilton *et al.* 2012), we hypothesized that ozone induction of plant defences against aphids is only effective in non-symbiotic plants. Since the fungal symbiont may be target of ozone-induced defence against biotrophic fungi (known to be elicited by the pollutant; Sandermann *et al.* 1998; Tamaoki 2008) or ozone-triggered oxidative stress in the apoplast, we hypothesized that the defensive mutualism conferred by the endophyte is impaired under ozone. Both outcomes will ultimately result in decreasing the relative fitness of symbiotic vs. non-symbiotic plants or, in other words, the mutualism effectiveness.

Materials and methods

BIOLOGICAL MATERIAL

We worked with Italian annual ryegrass *Lolium multiflorum* Lam. that establishes a persistent symbiosis with the fungal endophyte *Epichloë occultans* (Moon *et al.* 2004). *L. multiflorum* is a high-quality forage grass native to Mediterranean and naturalized in the Argentinean Pampas, where populations have been found to present high frequency of endophyte symbiotic individuals (Gundel *et al.* 2009). Our previous work indicates that symbiotic plants are chemically protected against phloem-sucking herbivores such as aphids (Omacini *et al.* 2001; Gundel *et al.* 2012). In addition, *L. multiflorum* has shown to present genetic variation in the response to stress by ozone (Martínez-Ghersa, Olszyk & Radosevich 2008).

Seeds of *L. multiflorum* were collected from an old-field vegetative community in Inland Pampa sub-region (Province of Buenos Aires, Argentina). A preliminary assessment indicated a high frequency (95%) of endophyte symbiotic seeds. The non-symbiotic biotype was generated by subjecting half of the seeds to the systemic fungicide triadimenol (β-(4-chlorophenoxy)-α- (1,1-dimethylethyl)-1H-1, 2,4-triazole-1-ethanol) (Baytan, Bayer, Buenos Aires, Argentina; 5 mg g^{-1} seed). Fungicide-treated (E−) and untreated (E+) seeds were sown in contiguous 1 m^2 plots in an experimental field (School of Agriculture, University of Buenos Aires) to increase the number of seeds. Cross-pollination was allowed to prevent genetic segregation between the biotypes. This cultivation was repeated for four growing cycles (years). The seed lots harvested at the end of the fourth growing season (F4) were characterized in terms of the proportion of endophyte symbiotic seeds and were used in the experiments presented in this article. The symbiotic status was checked by observing stained seeds under microscope, magnification 10× and 40× (Latch, Potter & Tyler 1987) and they presented contrasting frequencies of symbiotic seeds (E− = 5%, and E+ = 95%, *n* = 100).

Aphids (*Rhopalosiphum padi*; Hemiptera: Aphididae) were collected in the vicinity of the School of Agriculture and reared on 4-week-old wheat plants (*Triticum aestivum* L.) in a greenhouse with controlled temperature, photoperiod and radiation (25 °C, 14/10 day/night, and 1888 Mj m^{-2} h^{-1}). The wheat plants were grown in 1 L pots filled with the same substrate that was used for the experiments (see below); wheat plants on which aphids were reared, were replaced weekly by new ones. This allowed us to maintain and increase the aphid population for subsequent use.

EXPERIMENTS

Two experiments were carried out to evaluate the effect of episodic exposure to high ozone concentration on plant-herbivore interaction mediated by the symbiosis with fungal endophytes.

The experiments were conducted in spring (September–November in the Southern hemisphere; 34° 35′ S, 58° 35′ W), in 2010 and 2011. Endophyte-symbiotic (E+) and non-symbiotic (E−) *L. multiflorum* plants were grown individually in 0·5 L pots filled with equal parts of soil, sand and peat. These pots were placed outdoors in a common garden and watered regularly. Two to three seeds were directly sown in a pot and after seedling emergence and confirming their symbiotic status, each pot was thinned to one plant. The symbiotic status of the seedlings was checked by looking for the endophyte in the first 2–3 mm of the stained outer leaf under microscope, magnification 10× and 40× (Belanger 1996).

When plants had between 4–5 tillers and 3–4 leaves per tiller, they were subjected to episodes of high ozone concentration or kept at ambient ozone concentration (see below). During two consecutive days, plants were exposed to ozone in open-top chambers during 4 h coincident with the natural daily peak of ozone (at noon). The vast majority of studies have looked at constant and continuous exposure to ozone determining chronic conditions that mirror future environmental scenarios (Krupa & Manning 1988), as opposed to acute ozone exposure like the one used in our experiments. However, damage inflicted on plants by ozone under chronic and acute exposure, involves different defence strategies. Chronic damage, somewhat similar to a premature senescence, follows disturbances in sugar metabolism, inhibition of photosynthesis, imbalances in the redox-status and ROS-production in the chloroplast stroma (Vahala *et al.* 2003). In turn, we were interested in inferring acute damage, which resembles the hypersensitive response occurring after a pathogen or herbivore attack, the stress factor of our interest, and may interfere with the signalling pathway elicited by them (Kangasjärvi *et al.* 1994; Overmyer *et al.* 2005; Pineda *et al.* 2013).

Eight circular open-top chambers of about 1·5 m diameter and 2·5 m height with crystal PVC walls mounted on a metal structure were used. Level of ozone and temperature in each chamber was controlled. Ozone was generated by a spark discharge-type ozone generator (Hogsett, Tingey & Holman 1985) that increases the ozone concentration up to ≈120 ppb in the 'high-ozone' treatment. On the other hand, the 'low-ozone' treatment consisted in ambient air pumped through an activated charcoal filter (≈1·5 ppb). Ozone was continuously monitored using a Model 450 Ozone Monitor API-Teledyne Instrument (Teledyne Advanced Pollution Instrumentation, San Diego, CA, USA).

Eight plants (4 E+ and 4 E−) were placed in each chamber for ozone treatment. After that, the plants were moved back to the common garden and randomly distributed. Half of the E+ and E− plants coming from each chamber were randomly assigned to the herbivory treatment which consisted in placing 10 aphids on each. Each plant was covered with a white voile bag structured with a plastic tubular net, to avoid aphid migration. The population size of aphids on each plant was determined by counting them periodically for 21 days. Plants that were not infested with aphids were subjected to the same manipulation to maintain conditions constant and for checking eventual infestations of aphids. Aphids were removed after 21 days and stored in 3 mL eppendorf (one per plant) for further measurements. At harvest, plant shoot and root biomass was determined by placing material separately in an 60–70 °C oven for 48 h, and weighed. In the experiment carried out in 2010, parts of these shoots (leaf blades and pseudostems) were lyophilized to evaluate the levels of loline alkaloids. The content of three loline derivatives (NANL: N-acetilnorloline; NFL: N-formylloline; and NAL: N-acetylloline) was determined as was total content (NANL + NFL + NAL). This was analysed by capillary gas chromatography with a flame ionization detector (Blankenship *et al.* 2001). Concentration of alkaloids was expressed as alkaloid (μg) per unit of plant material (g). In 2011, a

sample of 100 aphids per plant was taken and individuals were classified in different life cycle instars: nymph, apterous and winged adult (Patch 1917). Two variables were determined for each category: number of aphids per plant, and aphid mass (in mg) of each instar per plant. The weight was estimated by taking five subsamples of 20 aphids per category and calculating the mean of the five groups.

DATA ANALYSIS

Statistical analyses were performed with R software (R Core Team 2013). Effects of endophyte and ozone on aphid population size was analysed with generalized linear mixed model (GLMM) fitted by maximum likelihood with plants (E+ and E−) nested within year (2010 and 2011) and year as random effect (lme4 package, glmer function). Poisson error distribution and a log-link function were used. Individual plant biomass was used as a covariate factor. Overdispersion was corrected by scaling with Pearson's χ^2 (Bolker *et al.* 2009) and the dispersion parameter was included in the model. Effects of the same two factors on aphid population structure which was only evaluated in 2011 were modelled with a generalized linear model with binomial distribution. Since proportion of aphids in each instar is based on a binary response variable (nymph/adult), we used a logit link function (stats package, glm function; Crawley 2007; Zuur *et al.* 2009). No overdispersion was observed. Given that endophyte and ozone effects on individual aphid weight may depend on whether it is a nymph and an adult, instar was considered as independent factor. For this latter analysis, we used a general linear model with the identity link function (stats package, function aov; Crawley 2007).

Effects of endophyte, ozone and aphid herbivory on total plants biomass and root/shoot ratio were modelled with linear mixed-effect model fitted by restricted maximum likelihood (REML) with year (2010 and 2011) as random factor. Gaussian distribution was used and number of tillers per plant as a covariate (nlme package, lme function). The variable year was modelled by varIdent variance structure, to meet ANOVA assumptions (Pinheiro *et al.* 2009). The level of loline alkaloids that was only measured in 2010 on endophyte symbiotic plants was analysed with ANOVA in which ozone and aphid were independent factors. Alkaloids were transformed by sqrt(y) to meet assumptions of normality and homogeneity of variance. Information reported in results is untransformed data.

Results

APHID POPULATION

The number of aphids per plant on day 21 in the experiment 2010 and 2011 was affected by the interaction effect between endophyte and ozone ($Z = 4.20$, d.f. = 1, $P < 0.001$; Fig. 1a,b). The enormous difference between aphid population sizes in E+ and E− plants not exposed to ozone was not observed in ozone-exposed plants (Fig. 1a,b; Table S1, Supporting information). Under low ozone condition, average number of aphids was 56 and 64% lower on symbiotic plants compared to that of non-symbiotic plants in 2010 and 2011, respectively (Fig. 1a,b). In both years, the population size of aphids was of equal size in both E+ and E− plants exposed to ozone (≈ 2998 in 2010, and ≈ 1553 in 2011).

The structure of aphid populations in terms of the proportion of nymphs and adults (apterous + winged) was interactively affected by the symbiotic status of plants and ozone exposure ($F_{1,28} = 780.64$, $P < 0.001$; Fig. 2a; Table S2). While proportion of nymphs was clearly higher on non-symbiotic plants than on symbiotic ones under low-ozone treatment (E−: 80% vs. E+: 14%), the opposite was observed after exposing plants to an episode of high ozone level (E−: 32% vs. E+: 59%). Under low-ozone, aphid populations were characterized by a higher proportion of adults on endophyte symbiotic plants compared to non-symbiotic ones, a pattern that was modified by the treatment of high-ozone (Fig. 2a; Table S2).

The endophyte fungus effect on individual weight of nymphs and adults depended on the ozone level ($F_{1,56} = 47.66$, $P < 0.001$; Fig. 2b; Table S3). The differences in average body weight of insects at both instars, between symbiotic and non-symbiotic plants were much higher under low ozone treatment (Fig. 2b). On average
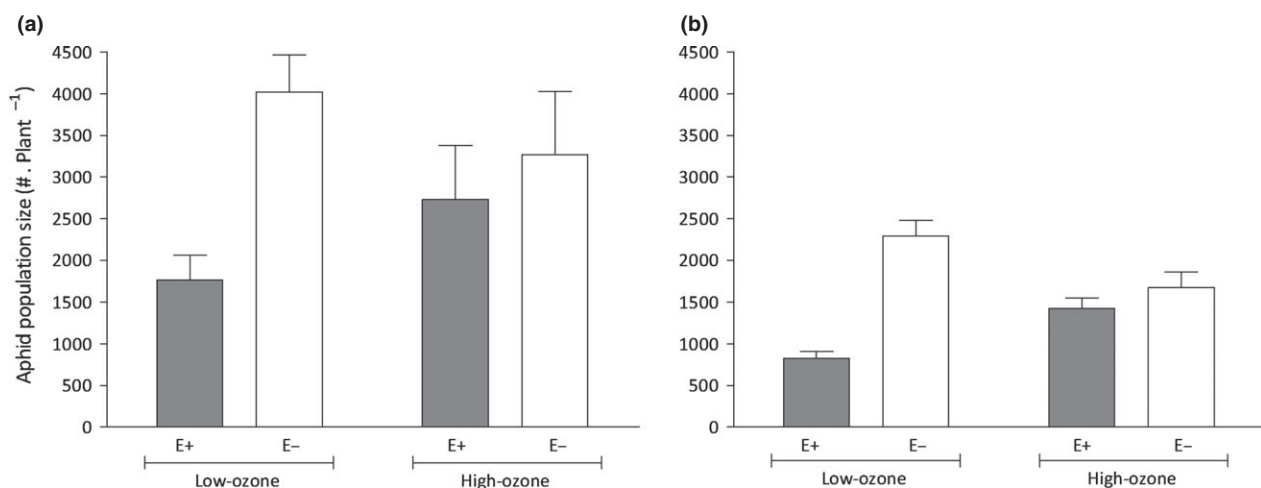

Fig. 1. Population size of aphids (*Rhopalosiphum padi*) in *Lolium multiflorum* plants symbiotic (E+) and non-symbiotic (E−) with the fungal endophyte *Epichloë occultans*, recorded 21 days after the ozone treatment [episodic exposure to low (Low-ozone) and high (High-ozone) ozone concentration]. Values are mean ± SE of n = 4 and n = 8, in the experiments conducted in 2010 (a) and 2011 (b), respectively.
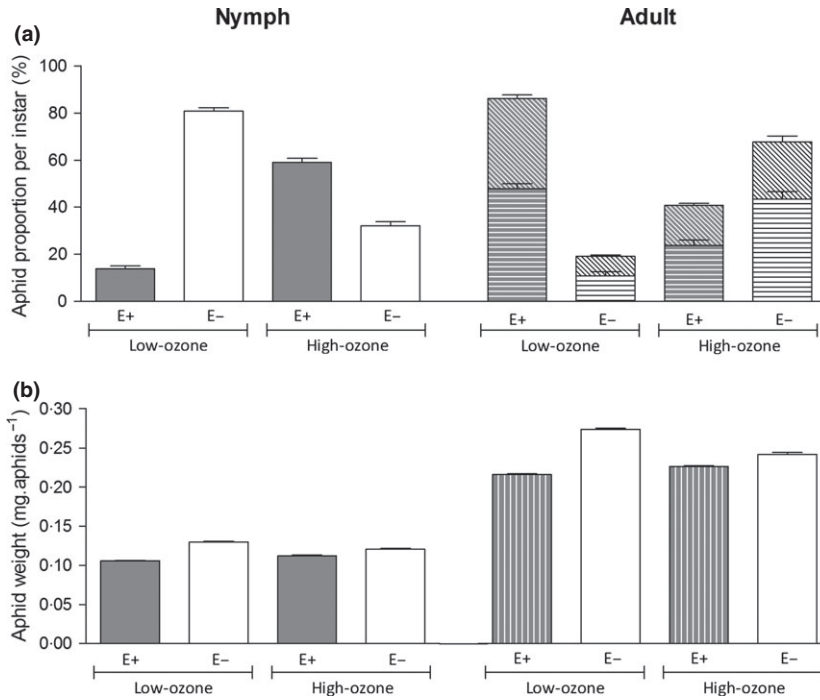
Fig. 2. Population structure (a) as percentage of aphids in each instar [nymph and adults (apterous + winged)] and individual weight (b) per instar [nymph and adults (average of apterous and winged)] of the aphid *Rhopalosiphum padi* growing on *Lolium multiflorum* plants symbiotic (E+) and non-symbiotic (E−) with fungal endophyte *Epichloë occultans*, recorded 21 days after the ozone treatment [episodic exposure to low (Low-ozone) and high (High-ozone) ozone concentration]. Bar of adults in (a) shows differentially the proportion of apterous (diagonal striped pattern) and winged (straight striped pattern). The individual weight of adults in (b) includes apterous and winged (vertical striped pattern). Values are mean ± SE of $n = 8$.

for the two instars –nymph and adult–, the endophyte presence reduced the weight of individual aphids by about 20 and 6% under low and high ozone treatment, respectively (Fig. 2b).

PLANT BIOMASS

In 2010 and 2011, individual plant biomass was affected by endophyte ($F_{1,86} = 5.86$, $P = 0.017$) and aphid ($F_{1,86} = 14.08$, $P < 0.001$), and irrespective of ozone treatment ($F_{1,86} = 0.01$, $P = 0.902$; Table S4). While dry weight biomass of symbiotic plants was higher than non-symbiotic plants, aphid herbivory had a negative effect on plant

growth (Fig. 3a,b). The root/shoot ratio was not affected by treatments in either year (data not shown).

ALKALOID CONTENT

Neither different loline derivatives individually (NANL, NFL and NAL) nor total level of loline alkaloids were affected by either ozone (Total alkaloid: $F_{1,12} = 0.35$, $P = 0.564$) or herbivory (Total alkaloid: $F_{1,12} = 0.06$, $P = 0.806$) in symbiotic plants (Tables 1 and S5). However, a strikingly variability among plants within the treatment combining ozone and herbivores was observed ($CV_{high-ozone \times aphid}$: 177.74 vs. $CV_{high-ozone \times no-aphid}$:
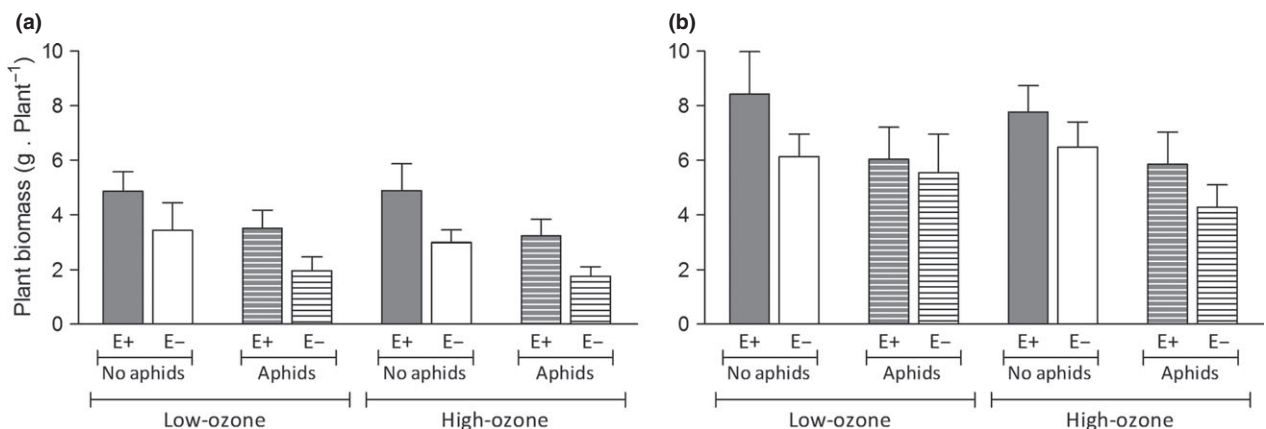

Fig. 3. Dry weight biomass (shoot + root) of *Lolium multiflorum* plant symbiotic (E+) and non-symbiotic (E−) with fungal endophyte *Epichloë occultans* recorded 21 days after the ozone treatment [episodic exposure to low (Low-ozone) and high (High-ozone) ozone concentration], and infested with the aphid *Rhopalosiphum padi*. Values are mean ± SE of $n = 4$ and $n = 8$, in the experiment 2010 (a) and 2011 (b), respectively.

Table 1. Level of the different loline derivatives (NANL: N-acetylnorloline; NFL: N-formylloline; and NAL: N-acetylloline) and total alkaloids (NANL + NFL + NAL) in *Lolium multiflorum* plants symbiotic with the fungal endophyte *Epichloë occultans*, 21 days after ozone treatment [episodic exposure to low (Low-ozone) and high (High-ozone) ozone concentration], and infestation by the aphid *Rhopalosiphum padi*. Units are $\mu g\ g^{-1}$, mean ± SE ($n = 4$)

Treatments		Loline alkaloid			
Ozone	Herbivory	NANL	NFL	NAL	Total
Low-ozone	No aphid	107 ± 23	183 ± 23	0 ± 0	289 ± 45
	Aphid	77 ± 11	132 ± 12	6 ± 4	220 ± 45
High-ozone	No aphid	72 ± 20	126 ± 24	3 ± 3	200 ± 46
	Aphid	63 ± 30	139 ± 50	2 ± 2	241 ± 91

90·17, $CV_{\text{low-ozone} \times \text{aphid}}$: 48·41, $CV_{\text{low-ozone} \times \text{no-aphid}}$: 87·69; CV: Coefficient of variation).

Discussion

The effectiveness of defensive mutualism conferred by vertically transmitted fungal endophytes to host plants was consistently reduced by ozone. Four hours of ozone exposure split in two consecutive days were enough to cause such an effect. The significant difference in the performance of aphids, in terms of population size, feeding on E+ plants vs. E− plants under low-ozone treatment vanished under high-ozone treatment. Similarly, ozone reduced the negative effect of the endophyte on aphid individual weight. But the greatest effect of ozone was on the structure of aphid populations; it completely shifted the proportion of instars by increasing and decreasing the number of nymphs in endophyte-symbiotic and non-symbiotic plants, respectively. This probably resulted from the co-occurrence of two processes, 1) efficacy decay of the endophyte-mediated mechanism of resistance against aphids and 2) increased resistance to herbivory in non-symbiotic plants. Even though the fungal alkaloids are considered the currency of the defensive mutualism, which may or may not be related to endophyte biomass (Wilkinson *et al.* 2000; Clay & Schardl 2002; Spiering *et al.* 2005; Rasmussen *et al.* 2007; Selosse & Schardl 2007; Schardl 2010), we did not detect any effect of early ozone exposure of plants on levels of different derivatives of loline alkaloids. However, any effect of ozone on the level of lolines cannot be ruled out because measurements were taken 21 days after the exposure. It is worth noting, that the level of lolines in our plants were very low in all treatments (Bultman, Bell & Martin 2004; Schardl *et al.* 2007). Although we did not measure fungal biomass within plants, two non-exclusive mechanisms could be negatively affecting the fungal symbiont and the benefits it provides: (i) induction of plants defences against biotrophic microorganisms and (ii) ozone-triggered oxidative stress in the apoplast. Since endophyte positively affected the biomass of plants while ozone had no effect on it, the observed pattern of results seems to be irrespective of plant size.

Although not studied as extensively as CO_2, ozone is a gas that is increasing in the troposphere and it has been found to modify the interaction of plants with other organisms and drive evolutionary changes (Holopainen 2002; Lindroth 2010; Landesmann *et al.* 2013; Pineda *et al.* 2013). Specific signalling pathways triggered by abiotic stress may interact with those elicited by microbes and affect plant-herbivore interaction (Pineda *et al.* 2013). Among the several ways ozone can affect biological interactions, few studies have tried to tease apart direct from indirect effects. In fact, due to its gaseous and highly dynamic nature, ozone can potentially have indirect effects on herbivores through changes in plant quality and defences' expression (Sandermann *et al.* 1998; Valkama, Koricheva & Oksanen 2007; Lindroth 2010). While high concentration of CO_2 may reduce the impact of herbivores on plants through a negative effect of the quality of tissues by increasing C/N ratio (or nitrogen-limited diet), ozone may have an opposite effect by impairing photosynthesis (low C/N ratio) and cell structures (high availability of carbohydrates; Lindroth 2010). In our experiments, however, the opposite was evident since, at least in non-symbiotic plants, ozone did not result in bigger aphid population sizes. Increased susceptibility to aphids due to ozone was apparent in symbiotic plants. All this suggests that the pollutant might have changed the plant-herbivore interaction through different mechanisms in endophyte-symbiotic and non-symbiotic plants. It has been demonstrated that ozone can elicit hormonal plant defence signalling mechanisms and trigger the synthesis of antioxidants, some of them (phenolics) with anti-herbivory effects (Sandermann *et al.* 1998; Holopainen 2002; Lindroth 2010). As shown by Simons, Bultman & Sullivan (2008), the exposure of plants to methyl jasmonate was effective in inducing resistance to herbivores in non-symbiotic plants, but it hindered the resistance provided by the endophyte, apparently by reducing loline biosynthesis. Considering the facts that (i) symbiotic plants were bigger than non-symbiotic ones, (ii) ozone did not modify the size of the plants and (iii) the endophyte has usually small or null effect on tissue N-concentration (Omacini *et al.* 2001, 2009), our results suggest that the ozone effect on the grass-endophyte symbiosis could be mediated by any hormonal plant defence mechanism (salicyclic acid or methyl jasmonate).

Ozone effects on plant-herbivory interactions have been mostly studied by subjecting whole systems (plants and herbivores) at once, to high concentrations of the pollutant. Unlike results from Aspen FACE experiment where the ozone was applied over the whole community and affected both plants and herbivores (see Awmack, Harrington & Lindroth 2004; Mondor, Awmack & Lindroth 2010), plants in our experiments were exposed to ozone before aphid infestation. By evaluating the indirect effects of ozone on insects' performance, we found that its effect may be positive or negative depending on whether the plant is endophyte-symbiotic or not. A recent study aimed at evaluating the direct effect of ozone on *Metopolophium*

dirhodum aphids under artificial diets showed that ozone increased mortality and decreased aphid dispersion ability compared to the control treatment (Telesnicki *et al.* 2005). The impact of ozone direct effect on aphids could be important in natural conditions since aphids move within and between plants. However, it is difficult to predict the outcome of ozone when direct and indirect effects occur simultaneously, and it is possible that aphid infestation levels in polluted and unpolluted areas may remain similar. Aphid infestation and population growth dynamics are complex as they function as metapopulations. On the other hand, host plants may or may not be infected by a fungal endophytic symbiont. This habitat heterogeneity may modify the way in which ozone impacts and alters the direct and indirect effects on local aphid populations (Levine & Wetzler 1996).

A recent review concluded that responses to ozone at a population level are less understood than those at the individual level (Lindroth 2010). Here, we showed that ozone modified the effects of the endophyte both at the individual (aphid weight) and population level (size and structure). However, consequences on population features were more complex than that on the individual characteristics. While populations of aphids feeding on endophyte-symbiotic plants and unexposed to ozone were characterized by a very low proportion of nymphs (a typical feature of an aged population), aphid populations feeding on symbiotic plants but exposed to ozone presented a higher proportion of nymphs. On the other hand, the very high proportion of nymphs in aphid populations feeding on non-symbiotic plants not exposed to ozone (control situation) was reduced by ozone accompanied by a concomitant increment in adult instars (apterous and winged). Given that population size correlated positively with proportion of nymphs (data not shown), all the latter results clearly complement themselves and help to explain the pattern observed in the response variable population size. Finally, as winged aphids may appear in populations that had perceived a negative stimulus due for example, to food deprivation (low carrying capacity) or the presence of a poisonous compound (Müller, Williams & Hardie 2001; Mehrparvar, Zytynska & Weisser 2013), ozone seems to have a differential effect on endophyte-symbiotic plants and non-symbiotic ones increasing and decreasing the susceptibility to sap-sucking herbivores, respectively.

In conclusion, we showed that ozone can affect the short-term stability of the symbiotic interaction between an epichloid fungal endophyte and an annual grass, mainly through what is considered the basis of the mutualism, 'the defence against herbivores'. However, the resistance to aphids in symbiotic plants seemed not to be related to the alkaloids, the assumed currency of the mutualism, suggesting that ozone has affected other unknown compounds. Thus, ozone appears as a novel factor likely challenging the persistence of vertically transmitted fungal endophyte in host grass populations. Although it clearly merits further research, the increased incidence of ozone as a novel stress factor under some global chance scenarios could change the rules of certain symbiotic interactions in nature.

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Data accessibility

Data deposited in the Dryad Digital Repository http://dx.doi.org/10.5061/dryad.20bm4 (Ueno *et al.* 2015).

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Supporting Information

Additional Supporting information may be found in the online version of this article:

Appendix S1. Protocol for alkaloid determination.

Table S1. Summary of generalised linear mixed model relating the effect of endophyte (E+ and E−), ozone (Low- and high-ozone), and their interaction on aphid population size in both experiments (2010 and 2011) with individual plant biomass as covariate.

Table S2. Analysis of deviance (*F* test) showing the effects of endophyte (E+ and E−), ozone (Low- and high-ozone) and their interaction on the proportion of aphids in a given instar (nymph or adult) in experiment 2011.

Tabla S3. Analysis of Variance (ANOVA) of the effect endophyte (E+ and E−), ozone (Low- and high-ozone), aphid instar (nymph or adult) and their interactions on individual weight in experiment 2011.

Table S4. Summary of generalised linear mixed model relating the effect of endophyte (E+ and E−), ozone (Low- and high-ozone), aphid (aphid and no aphid) and their interactions on plant biomass in both experiments (2010 and 2011).

Table S5. Analysis of Variance (ANOVA) of the effect of ozone (Low- and high-ozone) and aphid (aphid and no aphid) on total alkaloid content (NANL + NFL + NAL) in experiment 2010.