Tittle: Ozone degrades floral scent and reduces pollinator attraction to flowers

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This is the accepted version of the following article: Farré Armengol, Gerard, et al. "Ozone degrades floral scent and reduces pollinator attraction to flowers" in New phytologist, vol. 209, issue 1 (January 2016), p. 151-160, which has been published in final form at DOI 10.1111/nph.13620

SUMMARY

• In this work we analyzed the degradation of floral scent volatiles from *Brassica nigra* by reaction with ozone along a distance gradient and the consequences for pollinator attraction.

• For this purpose we used a reaction system comprising three reaction tubes where we conducted measurements of floral volatiles by PTR-TOF-MS and GC-MS. We also tested the effects of floral scent degradation on the responses of the generalist pollinator *Bombus terrestris*.

• The chemical analyses revealed that supplementing air with ozone led to an increasing reduction in the concentrations of floral volatiles in air with distance from the volatile source. The results reveal different reactivities with ozone for different floral scent constituents, which emphasizes that ozone exposure not only degrades floral scents, but also changes the ratios of compounds in a scent blend. Behavioral tests revealed that floral scent was reduced in its attractiveness to pollinators after it had been exposed to 120 ppb O_3 over a 4.5 m distance.

• The combined results of chemical analyses and behavioral responses of pollinators strongly suggest that high ozone concentrations have significant negative impacts on pollination by reducing the distance over which floral olfactory signals can be detected by pollinators.

Keywords: *Brassica nigra*, *Bombus terrestris*, monoterpenes, anisaldehyde, phenol, p-cymene, behavioral tests.

INTRODUCTION

Volatile organic compounds (VOCs) mediate several ecological interactions between plants and other organisms (Dudareva *et al.*, 2006; Dicke & Baldwin, 2010). One of the ecological interactions mediated by VOCs is the communication between entomophilous plants and their respective pollinators (Farré-Armengol *et al.*, 2013). The establishment of such an interaction relies on plants producing chemical scent cues that can be identified by pollinators and facilitate communication over scales ranging from short to long-distance. These chemical cues can provide diverse information to pollinators, such as the species to which they belong, the availability and quality of rewards (Howell & Alarcón, 2007; Wright *et al.*, 2009), flower ontogeny (Mactavish & Menary, 1997; Goodrich *et al.*, 2006) and pollination state (Negre *et al.*, 2003). Floral scent cues also serve pollinators in their quest to locate the emitting source (flower) via scent trails that occur with concentration gradients (Cardé & Willis, 2008; Riffell *et al.*, 2008).

Ozone is a powerful oxidizing agent and a common atmospheric pollutant in the lower atmosphere that may react with and disturb these floral scents. Tropospheric ozone concentration has significantly increased since pre-industrial era times due to anthropogenic activity (IPCC, 2001, 2007, 2013), and it is predicted to increase more in the next decades, enhanced by global warming and changes in land cover (Val Martin *et al.*, 2014). Ozone has direct harmful effects on many living organisms including plants and animals (Mcgrath *et al.*, 2001; Kampa & Castanas, 2008; Díaz-de-Quijano *et al.*, 2012). Ozone can have significant negative impacts on plant reproductive success via its negative impacts on plant tissues and plant physiology (Bergweiler & Manning, 1999; Black *et al.*, 2007). Furthermore, many recent studies have reported that ozone and other common oxidative pollutants, such as hydroxyl and nitrate radicals, affect the emissions of VOCs from plants and the interactions they mediate (Pinto *et al.*, 2007a, 2010; McFrederick *et al.*, 2009; Blande *et al.*, 2010, 2011; Fuentes *et al.*, 2013). Tropospheric ozone can affect plant emissions and their effectiveness in two ways: first, by affecting plant physiology and inducing changes in the emission profiles (Andermann *et al.*, 1999; Peñuelas & Llusia, 1999; Holopainen & Gershenzon, 2010), and second, by mixing and reacting with the emitted compounds once they are released (Holopainen & Blande, 2013; Blande *et al.*, 2014).

The oxidative degradation of the VOCs emitted by flowers may reduce their concentration in an odor plume, decreasing the distances they can travel before reaching concentrations that are not detected by foraging pollinators (McFrederick *et al.*, 2008). Moreover, the reactivity of the individual VOCs in a blend differs both with the properties of the chemical and the properties of the oxidizing agent. Therefore, VOCs in a chemical blend may be degraded at different rates in ozone polluted (Atkinson & Arey, 2003) or in diesel fume (NO and NO_2) polluted environments (Girling *et al.*, 2013), leading to changes in the original ratios of VOC in the floral scent (McFrederick *et al.*, 2009). The oxidative reactions of ozone with plant-emitted VOCs lead to the formation of new organic compounds that can be volatile and persistent in the altered volatile blend (Pinto *et al.*, 2010). These *de novo* produced compounds are not part of the original scent of the species, and may induce confusion in the signal receivers, in this case pollinators, if they are able to detect its presence. All processes involving the reaction of ozone with VOCs may reduce the intensity of floral scent and provide significant additional variability to flower olfactory signals once they have been released, potentially with negative effects on the reliability of floral scent as an attractant.

The objective of this work was to analyze the effects of exposure to different ozone concentrations on the floral scent of *Brassica nigra*, while testing the effects of induced changes on the attraction of the generalist pollinator *Bombus terrestris*. The sensory abilities of bumblebees and their learning and memory capabilities are well known, which makes them one of the most suitable models for conducting behavioral studies (Chittka & Raine, 2006; Riveros & Gronenberg, 2009). *Bombus terrestris* is one of the most abundant and widespread bumblebee species in the West Palearctic and has a very relevant role as a pollinator in wild and cultivated plant communities (Rasmont *et al.*, 2008). The flower foraging preferences of *B. terrestris* display a large degree of generalism, which makes them a good pollination vector for a wide range of entomophilous plant species (Fontaine *et al.*, 2008). We expected floral scent to suffer quantitative and qualitative changes when exposed to ozone-enriched ambient air. We hypothesized (1) that floral scents would experience a greater degree of degradation with increasing distance from the scent source under higher ozone concentrations. We also hypothesized (2) that floral VOC mixtures might experience qualitative changes due to variation in the relative ratios of the existing compounds due to differences in their reactivity times with ozone, and also due to the formation of new compounds resulting from oxidative reactions of VOCs with ozone. With respect to flower-pollinator communication, we hypothesized (3) that pollinators would be more attracted to floral scent when it had not been exposed to ozone, than after being exposed to ozone-enriched ambient air over the longer distances tested.

MATERIALS AND METHODS

Brassica nigra plants and flower collection

The experiments were conducted from June to July 2014 at the University of Eastern Finland's Kuopio Campus. *Brassica nigra* (L.) W.D.J. Koch plants were grown from seed harvested from wild populations at sites near Wageningen University, the Netherlands. Plants were grown individually in 1 L plastic pots filled with a 3:1 mix of peat and sand and grown under greenhouse conditions with an approximate regime of light/dark cycle: 18h/6h, day temperature 23°C and night temperature 18°C and relative humidity 60%-80%. The plants were watered daily and fertilized with 0.1% 5-Superex (N:P:K 19:5:20) (Kekkilä, Finland) twice per week. Seeds were sown weekly to yield a constant supply of flowering plants (20 per week) throughout the experimental period. On each sampling day a bunch of inflorescences were cut at the greenhouse, put into a glass with water and transported to the lab for chemical measurements and/or behavioral tests.

Chemical measurements

Experimental design

We exposed the flower VOC emissions to 3 different ozone concentrations, 0, 80 and 120 ppb. For each ozone concentration tested, we measured VOC concentrations with a PTR-TOF-MS at 4 distances from the scent source within the reaction system (0 m, 1.5 m, 3 m and 4.5 m) (Figure 1). We repeated the measurements of VOC concentrations with eight different batches of flowers (weighing 1−2.5 g dry weight). We also sampled floral volatiles with adsorbent-filled tubes for each concentration and distance (n = 2-4) and analyzed them by GC-MS. We used STATISTICA version 8.0. (StatSoft, Inc., Tulsa, USA, 2007), to conduct general linear models testing the effect of ozone concentration and distance on floral VOC concentrations and also on the relative ratios of terpenes.

Ozone reaction system

We used an ozone reaction system comprising three glass tubes of 1.5 m length and 5.5 cm inner diameter that were connected in sequence with metal tubes of 4 mm inner diameter. The system allowed the collection of air at 4 different distances from the emission source (Figure 1). We used an activated carbon filter to clean the air entering the system of any VOCs. The cut flowers were put into a sealed glass jar where an incoming clean air flow of 900 mL min^{-1} was regulated with a mass flow controller (Alicat Scientific, AZ, USA). The clean air was mixed with floral volatile emissions inside the jar and was directed to the reaction system through Teflon tubing. Just before the entrance to the first reaction chamber, a tube connected to an ozone generator (Stable Ozone Generator, SOG-2; UVP, LLC-Upland, CA, USA) and carrying ozone enriched air at a mass flow controller regulated rate of 50 mL min^{-1} was joined to the tube carrying the floral volatile emissions. The first port from which air samples could be taken for chemical measurements and behavioral tests was situated just after the point that the two inlet flows mixed. The first port was named “distance 0”, after which the reaction system continued with three sequential reaction chambers, with further ports at the end of each chamber (distances 1, 2 and 3, at 1.5 m, 3 m and 4.5 m respectively) and an outlet at the end connected to an ozone scrubber. We used Teflon tubes of 4 mm inner diameter to connect the pump, the VOC filter, the ozone generator and the flower jar to the reaction system. We used an Ozone analyzer (Dasibi 1008-RS;

Dasibi Environmental Corp., Glendale, CA, USA) to calibrate and check the ozone concentrations achieved inside the reaction system.

PTR-TOF-MS measurements

A high-resolution proton-transfer reaction time-of-flight mass spectrometer (PTR-TOF-MS 8000, Ionicon Analytik, Innsbruck, Austria) was used to monitor floral VOC concentrations. Sample air from the chamber was introduced into the PTR drift tube via a 1.5 m length (outside diameter 1/16 inch) of heated (60°C) PEEK tubing at a flow rate of 200 mL min^{-1}. Hydronium ions (H3O+) were used as reagent ions to ionize organic compounds. The PTR-TOF-MS was operated under controlled conditions (2.3 mbar drift tube pressure, 600 V drift tube voltage and 60°C temperature). The raw PTR-TOF data were post-processed with the PTR-MS Viewer program (Ionicon Analytik). Concentrations were calculated by the program using a standard reaction rate constant of 2×10^{-9} cm^3 s^{-1} $molecule^{-1}$.

Volatile collection and GC-MS measurements

We collected air from each of the sampling ports into adsorbent-filled tubes for a more detailed analysis of the floral terpene emissions by GC-MS. The tubes were filled with adsorbents Tenax® and Carbopack™ (150 mg each; Markes International, Llantrisant, RCT, UK). A sampling air flow of 200 mL min^{-1} and sampling times of 30−40 min were used. The VOC samples were analysed by a GC-MS system (Agilent 7890A GC and 5975C VL MSD; New York, USA) with an approximate detection limit of 3 ng/mL. Trapped compounds were desorbed with an automated thermal desorber (TD-100;

Markes International Ltd, Llantrisant, UK) at 250˚C for 10 min, cryofocused at -10˚C and then transferred in a splitless mode to an HP-5 capillary column (50 m × 0.2 mm; film thickness 0.33 µm). Helium was used as a carrier gas. Oven temperature was held at 40˚C for 1 min, then programmed to increase by 5˚C min^{-1} to 210˚C, and then by 20˚C min^{-1} to 250˚C under a column flow of 1.2 mL min^{-1}. The column effluent was ionized by electron impact ionization at 70 eV. Mass spectra were acquired by scanning from 35-350 m/z with a scan rate of 5.38 scan/s.

Testing the responses of pollinators

Bombus terrestris

For the behavioral tests we used the bumblebee, *Bombus terrestris*, which was obtained as a group of three colonies each with a queen and providing an estimated 350-400 individuals, including adult workers, pupae, larvae and eggs (TRIPOL, Koppert Biological Systems, Netherlands). The bumblebees were kept in two conjoined ventilated polycarbonate cages giving a total foraging area of 1.4 m × 1 m × 0.7 m. The box containing the bumblebee colonies was put in one cage and the other cage was used to provide *Brassica nigra* flowers and a 50% sucrose solution to feed the bumblebees. We regularly provided fresh *Brassica nigra* flowers to familiarize the bumblebees with the floral scent and associated reward. The colonies remained in healthy condition and provided adult individuals that were suitable for behavioral tests throughout the 1 month period of the behavioral study.

Experimental design

We conducted behavioral tests to assess the preferences of *B. terrestris* presented with the three following odour combinations:

1. "*floral scent from distance 0 at 0 ppb O_3*" vs. "*clean air*" (n = 21).
2. "*floral scent from distance 3 at 120 ppb O_3*" vs. "*clean air*" (n = 24).
3. "*floral scent from distance 0 at 120 ppb O_3*" vs. "*floral scent from distance 3 at 120 ppb O_3*" (n = 21).

Floral scent sources were channeled from the port of the ozone exposure system corresponding with the distance and ozone treatment. The clean air comparison was first filtered and then passed through a glass jar with a pot of water to best match the humidity of the air exiting the reaction tubes. We conducted χ^2 tests to analyze the existence of pollinator preferences between compared air samples. We used paired t-tests to compare pollinator visitation between the artificial flowers of compared air samples.

Behavioral chamber

Behavioral tests were conducted in a cylindrical chamber made of transparent polycarbonate with a 1 m height and 1.5 m diameter (Figure 2). The lateral walls of the chamber were covered with light green paper to avoid interferences in bumblebee behavior due to visual interferences from outside the chamber. Two lamps were used as a light source and were positioned on the top of the behavioral chamber one on each side. The chamber had a 20 cm × 30 cm window at a central point in the side. Two metal tubes of approximately 1 m length and 4 mm inner diameter were inserted into the cage entering from the top and positioned at opposite sides of the chamber. The metal tubes were connected to the two incoming air sources to be tested against each other inside the behavioral chamber. The metal tubes had some holes in the section, which released the odour sources close to artificial inflorescences that were placed in a metal support on the floor of the chamber. The artificial inflorescences consisted of yellow non-scented paper cut into the shape of petals and attached to a thin white Teflon tube with pins; the model resembled an inflorescence of *Brassica nigra*. Each inflorescence consisted of 8 flowers with position rotated around the tube. A third metal tube with the same dimensions was inserted in the center of the chamber. This tube had many holes all along its length oriented to all directions and was connected to a pump to draw air from the chamber (Figure 2).

Behavioral tests

Before starting the behavioral tests a series of checks and calibrations were conducted. First, the reaction system was turned on and outlet emissions were monitored by PTR-TOF-MS until a steady state was reached. After that we connected the two air sources that we wanted to test to the behavioral chamber. The pumps were turned on and the two incoming air flows were adjusted to 500 mL min^{-1} and the central outlet tube to 1 L min^{-1} (Figure 2). We then waited for another 30 minute period for the stabilization and homogenization of the air flows and VOC concentrations in the behavioral chamber system. For each test an individual bumblebee was collected from the colony in the dark and taken in a small pot to the adjacent lab where the behavioral chamber was housed. Each bumblebee was released from a central point of the chamber equidistant from the odour sources. At the start of the test the two lamps were turned on and the clock was started when the bumblebee started to fly. Each bioassay was observed continually for 10 minutes. The chamber was divided into two halves – one for each odour source – and the time spent in each half was recorded. When a bumblebee spent 315 seconds or more in one of the two halves, a choice for the respective odour source was assigned. However, when the times spent in each half differed in less than 30 seconds we determined that the test resulted in no choice. We also recorded the number of visits that the bees made to the artificial inflorescences. A visit was considered to have occurred when a flying bumblebee landed on one of the artificial inflorescences. Short flight movements between flowers within the same inflorescence were not considered to be different visits. If the bumblebees left the inflorescence, flew in the open chamber and landed again, we considered it a new visit. In addition, we transformed the data on pollinator visitation into a binary variable (0/1) for the statistical analyses. We assigned the value zero when no visits were conducted to artificial flowers during the test and we assigned the value one when pollinators conducted one or more visits. Once the test finished we released the bumblebees in a separate cage to avoid using the same individual for different test replicates on the same day, and we took a new bumblebee for the next trial.

RESULTS

Effects of ozone on the chemistry of floral emissions

Ozone concentration and distance from the floral scent source had a negative effect on the concentration of floral scent volatiles (Figure 3). Monoterpene (m/z 137.133), anisaldehyde (m/z 137.1562), and phenol (m/z 95.1194) concentrations showed very significant negative correlations with ozone concentration (P<0.0001), distance

(*P*<0.0001) and the interaction between ozone concentration and distance (*P*<0.0001). *p*-Cymene (m/z 135.1174) concentration also showed a very significant negative correlation with ozone concentration (*P*<0.0001) and distance (*P*=0.013). However, benzaldehyde (m/z 107.0497) concentration increased with ozone concentration (*P*=0.8) and distance (*P*=0.3), although the effects were not found to be significant (Figure 4).

Under the highest ozone concentration tested, at the longest distance from the scent source (4.5 m), monoterpene concentration decreased by 26.4%, anisaldehyde decreased by 27%, phenol decreased by 29.5%, *p*-cymene decreased by 31% and benzaldehyde increased by 17%. These compound-specific responses lead to changes in the relative composition of floral VOC blends. A detailed analysis of the composition of floral terpene emissions by GC-MS showed gradual changes with distance when exposed to ozone, although changes were not found to be significant (Figure 5). When exposed to increasing ozone concentrations the monoterpenes β-myrcene, β-thujene, (Z)-β-ocimene and γ-terpinene showed gradual relative increases with respect to other terpene compounds, while α-pinene gradually decreased.

Pollinator responses in behavioural tests

Bumblebees showed a clear orientation bias toward "*floral scent from distance 0 at 0 ppb* O_3" over "*clean air*" (χ^2 test, *P*=0.01) (Figure 6A). From a total of 21 tests, thirteen bumblebees spent more time in the half of the arena with "*floral scent from distance 0 at 0 ppb* O_3", three spent more time in the half with "*clean air*", and five individuals did not make a clear choice. Bumblebees showed no clear orientation bias when presented with "*floral scent from distance 3 at 120 ppb* O_3" and "*clean air*" (χ^2 test, *P*=0.37) (Figure 6B). From a total of 22 tests, eight bumblebees spent more time in the half with "*floral scent from distance 3 at 120 ppb O_3*", twelve of them spent more time in the half with "*clean air*", and two individuals did not make a clear choice. Finally, bumblebees showed a marked orientation bias toward "*floral scent from distance 0 at 120 ppb O_3*" over "*floral scent from distance 3 at 120 ppb O_3*" (χ^2 test, *P*=0.005) (Figure 6C). From a total of 21 tests, fifteen bumblebees spent more time in the half with "*floral scent from distance 0 at 120 ppb O_3*", three of them spent more time in the half with "*floral scent from distance 3 at 120 ppb O_3*", and three individuals did not make a clear choice.

Bumblebees made landings on artificial flowers in some of the tests conducted (Figure 7). The results show that more bumblebees landed on artificial flowers associated with "*floral scent from distance 0 at 0 ppb O_3*" than on artificial flowers associated with "*clean air*" (paired t-test, *P*=0.04) (Figure 7A). More bumblebees landed on artificial flowers associated with "*floral scent from distance 3 at 120 ppb O_3*" than on artificial flowers associated with "*clean air*", but the difference was not significant (paired t-test, *P*=0.08) (Figure 7B). Finally, more bumblebees landed on artificial flowers associated with "*floral scent from distance 0 at 120 ppb O_3*" than on artificial flowers associated with "*floral scent from distance 3 at 120 ppb O_3*" (paired t-test, *P*=0.01) (Figure 7C).

DISCUSSION

Quantitative and qualitative changes in floral scents after exposure to ozone

The concentrations of floral VOCs were significantly reduced with increasing distance from source when exposed to ozone enriched ambient air. We started to observe degradation of the floral volatiles emitted by *B. nigra* at the lower ozone level tested (80 ppb) over a distance of 1.5 m. The highest degradation levels of 25 to 30% were observed at 120 ppb O_3 over a distance of 4.5 m. Ozone degradation of vegetative VOCs has been previously reported (Pinto *et al.*, 2007a, 2007b, 2010; Blande *et al.*, 2010; Li & Blande, 2015) but, to our knowledge this is the first work to provide experimental evidence and quantification of floral scent degradation with ozone exposure. McFrederick et al. (2008) previously published a theoretical work modeling the degradation of three common floral monoterpenes under different concentrations of ozone and hydroxyl and nitrate radicals, whose predictions are mostly in accordance with our results. Girling *et al.* (2013) empirically demonstrated that diesel exhaust fumes, which include oxidant pollutants other than ozone, such as NO_2, NO, CO and SO_2, degrade floral scent volatiles that play relevant roles in the stimulation of proboscis extension reflex in honeybees. Also, several previous works have examined the ozone degradation of vegetative VOCs and showed how this can interfere with, or even disrupt some other ecological interactions of plants (Pinto *et al.*, 2007a, 2007b; Blande *et al.*, 2010; Li & Blande, 2015).

Individual VOCs in the blend of floral volatiles showed varying degrees of degradation, which are explained by their different reactivities with ozone (Atkinson *et al.*, 1995; Atkinson & Arey, 2003). The range of different reaction rates with ozone displayed by VOCs in the floral scent blend suggests that ozone pollution will induce changes in the relative composition of floral blends and that these changes will increase with increasing distance from the volatile source. In fact, we detected some changes in the relative composition of terpenes in the floral scent with increasing ozone concentration and distance, although they were not found to be significant probably due to low statistical power (Figure 5).

Effects of ozone-related changes in floral scent on the attraction of pollinators

Our results on the behavioral responses of *B. terrestris* clearly indicate a reduction in orientation toward floral scent cues after they have been exposed to ozone. *B. terrestris* displayed a clear orientation bias towards unaltered floral scent over clean air (Figure 6A) and there were significantly more landings on the artificial flowers associated with that scent (Figure7A). This observation confirmed the usage of floral scent cues by *B. terrestris* and also set a baseline observation for our behavioral arena. We later compared the responses of *B. terrestris* to floral scent exposed to 120 ppb ozone over the longest distance of 4.5 m against clean air and pollinators showed no preference for either of the two options (Figures 6B, 7B). This clearly suggests that exposure of floral scent to high ozone concentrations led to a loss in attractiveness of the floral scent to pollinators. Finally, we compared the responses of *B. terrestris* presented with a choice of floral scent mixed with 120 ppb ozone at distances of 0 m and 4.5 m through the reaction chamber, and observed that pollinators clearly preferred the scent at the 0 m distance (Figure 6C) and visited the artificial flowers associated with it more frequently (Figure 7C), which strongly supports that attraction to floral scent is gradually reduced with distance under high ozone concentrations.

We observed a significant degradation of floral scent cues after exposure to ozone, which may explain the loss of attractiveness to pollinators. High ozone concentrations like those tested here may cause a significant reduction in the distance that floral chemical cues can travel before reaching concentration levels that are below the olfactory detection limits of pollinators. This may be translated into a significant reduction in the distance over which floral chemical cues can be utilized by pollinators. Previous work by Girling *et al.* (2013) demonstrated that primary pollutants in diesel exhaust can differentially degrade the volatiles emitted by oilseed rape flowers. They additionally showed that removal of the two most reactive compounds from the blend resulted in a loss of the proboscis extension reflex of conditioned honeybees. Although the blend modification tested was a little bit more extreme than those encountered upon natural degradation processes, the removal of those two reactive compounds provides a strong indication that floral blend alteration has an important impact on foraging behaviors. In this work, we showed that far more moderate alterations of the entire blend, not involving the full elimination of any specific component, result in a loss of attractiveness of the blend to pollinators.

Qualitative changes in floral scent composition may lead to disturbance of pollinator attraction to floral odor plumes (Beyaert & Hilker, 2014). The correct recognition of plant volatile cues by foraging insects depends not only on the presence of certain compounds or the magnitude of the whole signal, but also on the ratios of the compounds that constitute the volatile blend (Bruce *et al.*, 2005). The effects of qualitative changes in floral scents on the attraction of pollinators may depend on the reliance of pollinators on innate olfactive preferences and their olfactive learning capabilities (Cunningham *et al.*, 2004; Schiestl & Johnson, 2013). While specialist pollinators show innate preferences towards specific blends of volatiles that are typical of their host plants, generalist pollinators are capable of learning the floral scents of the plants in the community and associate them with their floral rewards (Raguso, 2008; Riffell, 2011; Riffell *et al.*, 2013). For this reason, it is important for reward-offering plants to maintain a good level of reliability in their floral signals for pollinators, through the maintenance of low levels of variability (Wright & Schiestl, 2009; Knauer & Schiestl, 2014). Such low levels of variability in floral traits have been postulated to be beneficial for reward-offering plants (Salzmann *et al.*, 2007). Pollinators promote the selection of uniformity in the olfactive and visual traits of rewarding flowers, due to the advantages that flower consistency bring to both pollinators (higher foraging efficiency) and plants (less deposition of heterospecific pollen on the stigmas) (Gegear & Laverty, 2005). The qualitative changes in the relative composition of floral volatile cues caused by ozone exposure can have significant negative impacts on the correct learning and recognition of floral olfactive signals by foraging pollinators.

Implications of floral scent degradation by increasing tropospheric ozone concentrations

The increase in tropospheric ozone since the start of the industrial era is estimated to be around 35% with subtle differences among regions (IPCC, 2001, 2007, 2013). Mean annual tropospheric ozone concentrations over the mid latitudes of the Northern Hemisphere currently range between 20 and 45 ppb (Vingarzan, 2004). However, ozone concentrations are significantly higher in some areas (Kleinman *et al.*, 2002), which can reach or surpass 120 ppb, the highest ozone concentration that we tested in our experiments. The effects revealed by our work may be especially relevant for those regions with high tropospheric ozone concentrations. Many insect species could be negatively affected by disruption of volatile chemical communication due to ozone pollution. In the case of pollinator species these effects would have major economic and ecological impacts. Among the plant communities experiencing the most relevant effects we may find agricultural lands close to urban areas to be reduced in pollination efficiency. The most important concerns arising from these results may include reduced crop productivity and the disruption of several ecological processes related with pollination in plant communities affected by ozone pollution.

Conclusions and future perspectives

Our results strongly suggest that ozone can have significant negative effects on pollinator attraction to flowers. High ozone concentrations in ambient air caused fast degradation of *B. nigra* floral scent with increasing distance from the scent source, reducing the range over which flowers can be identified by pollinators. Behavioral tests conducted with *B. terrestris*, a common and widespread generalist pollinator, confirmed that ozone concentrations of 120 ppb, which can frequently occur near big urban areas, can strongly inhibit pollinator attraction to flowers.

The effects of ozone on VOC mixtures emitted by plants have been explored in several studies and the implications for plant communication with other plants, herbivores and predators have been addressed, but the effect on air concentrations of floral VOCs has not. Therefore, further experiments to test the effects in other plant species are warranted. In addition to pollinator response tests, new experiments may also include estimates of pollination success and fruit/seed production to explore the effect of ozone exposure and the related changes in floral scent on plant reproduction.

ACKNOWLEDGEMENTS

This research was supported by the Academy of Finland decision numbers 256050, 251898 and 141053, Spanish Government grant CGL2013-48074-P, the Catalan Government grant SGR 2014-274, the European Research Council Synergy grant ERC-2013-SyG-610028 IMBALANCE-P, the COST Action FP1204 GreenInUrbs, and by the Air Liquide Foundation (AIRLICOVS grant).

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FIGURE CAPTIONS

Figure 1. Schematic of the ozone reaction system. Arrows indicate the direction of the air flow. A circled triangle represents the pump. Black boxes represent mass flow controllers.

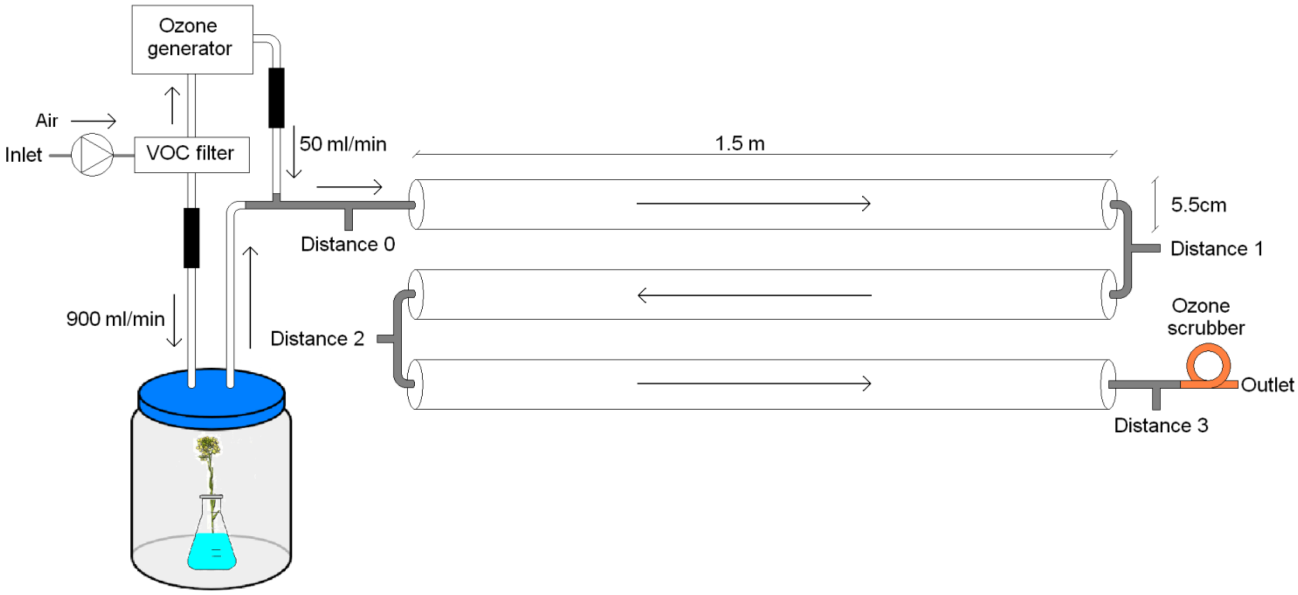

Figure 2. Behavioral test chamber. Arrows indicate the direction of air flow.

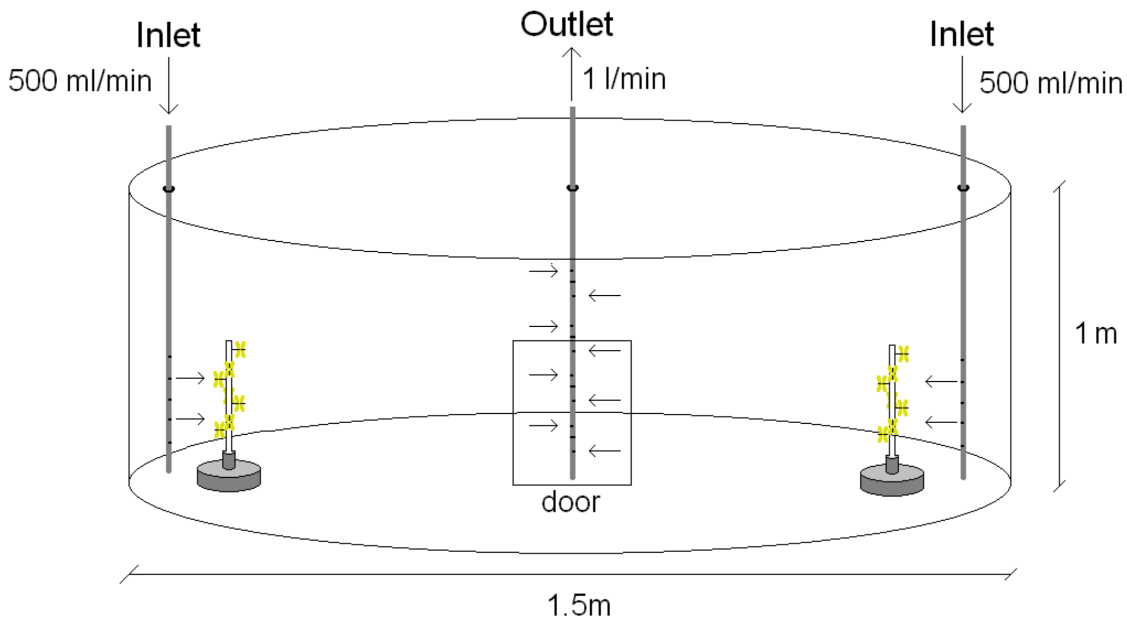

Figure 3. Floral scent degradation by ozone. The figure shows the relative decrease in monoterpene (m/z 137.133), anisaldehyde m/z (137.1562), phenol (m/z 95.1194) and *p*-cymene (m/z 135.1174) concentrations of *Brassica nigra* floral scent exposed to different ozone concentrations (0 ppb, 80 ppb, 120 ppb) at different distances from the emitter flower source (1.5 m, 3 m, 4.5 m) measured with PTR-TOFF-MS. Error bars indicate SEM (n = 8).

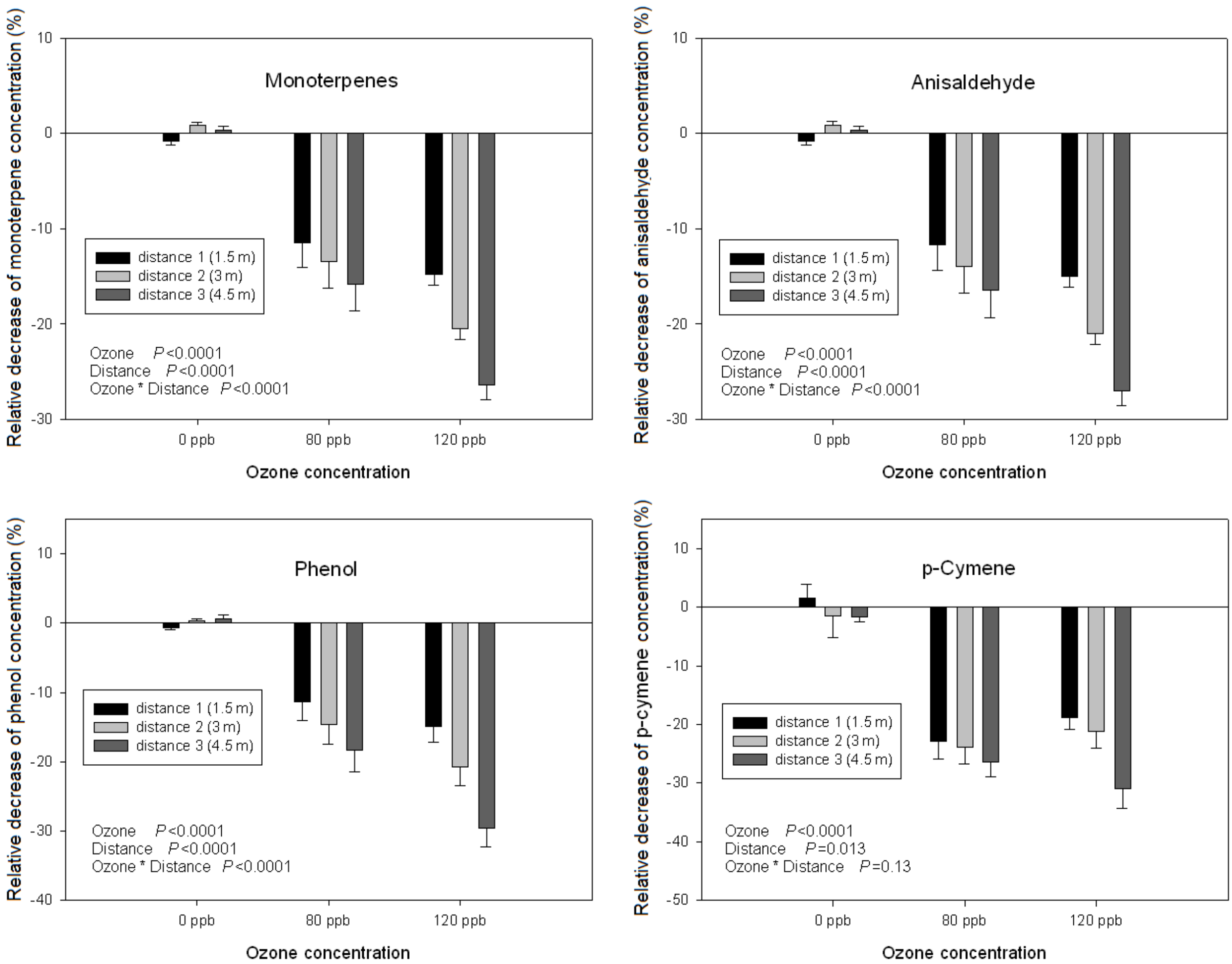

Figure 4. Relative increase in benzaldehyde (m/z 107.0497) concentrations of *Brassica nigra* floral scent exposed to different ozone concentrations (0 ppb, 80 ppb, 120 ppb) at different distances from the emitter flower source (1.5 m, 3 m, 4.5 m) measured with PTR-TOFF-MS. Error bars indicate SEM (n = 8).

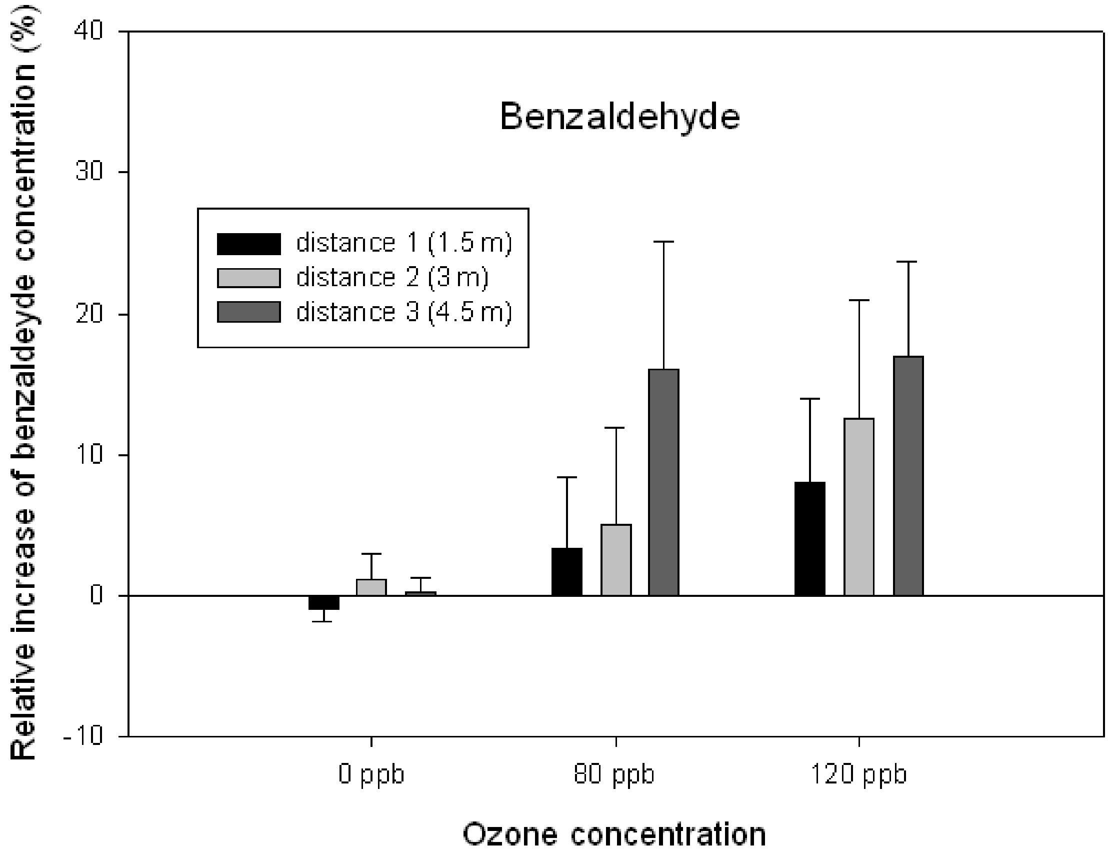

Figure 5. Relative floral terpene composition (%) at different distances from scent source under different ozone concentrations measured with GC-MS (n = 2, 3, 2, 2, 4, 2, 2, 4). Changes in the percentage of relative contribution of the different terpene compounds to the total terpene emissions were analyzed using general linear models, but no significant patterns of change were detected.

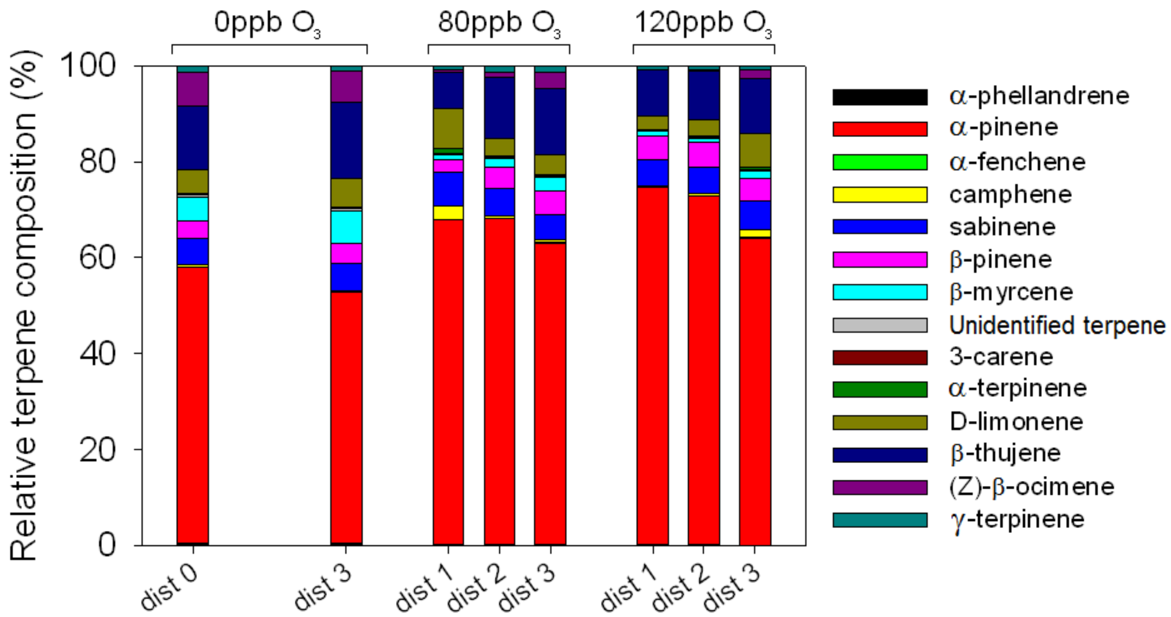

Figure 6. Pollinator orientation in choice tests comparing: A) floral scent (distance 0 at 0 ppb O_3) vs. clean air (filtered air with no flower scent) (*n*=21); B) floral scent (distance 3 at 120 ppb O_3) vs. clean air (filtered air with no flower scent) (*n*=24); C) floral scent (distance 0 at 120 ppb O3) vs. floral scent (distance 3 at 120 ppb O_3) (*n*=21). Asterisks indicate the level of significance of χ^2 tests (*P<0.05; **P<0.005).

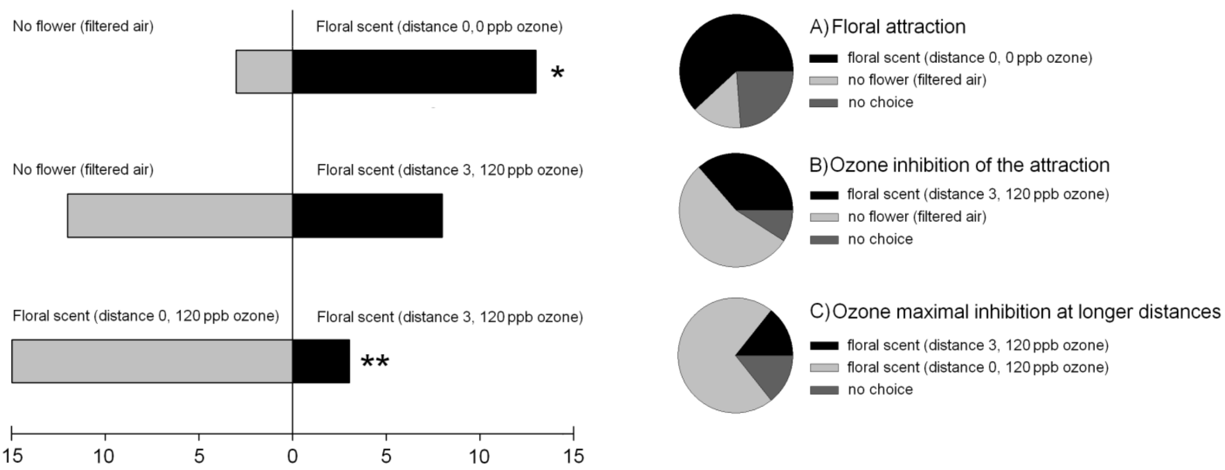

Figure 7. Pollinator visitation to artificial flowers for the behavioral tests comparing: A) floral scent (distance 0 at 0 ppb O_3) vs. clean air (filtered air with no flower scent) (n=21); B) floral scent (distance 3 at 120 ppb O_3) vs. clean air (filtered air with no flower scent) (n=24); C) floral scent (distance 0 at 120 ppb O_3) vs. degraded floral scent (distance 3 at 120 ppb O_3) (n=21). Asterisks indicate the level of significance of paired t-tests (*P<0.05). Error bars indicate SEM.