Assessing the potential of tropospheric oxidants to interfere with insect host location, mate location, and foraging choice

Brynn Sequoia Cook

Simi Valley, CA

B.S. in Environmental Sciences University of California Berkeley, 2013

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INTRODUCTION

Insects are critical to maintaining natural and managed ecosystems, functioning as pollinators, food sources, and organic matter recyclers (Yang et al. 2014; Losey and Vaughan 2006). Insects are particularly important to humans in their role as pollinators for managed agricultural systems (McGregor 1976; Klein et al. 2007). Insect pollinators contributed an estimated €100 billion in pollination services worldwide in 2005 (Gallai et al. 2009). However, despite their importance to ecosystems, food security, and the economy, insects have recently experienced alarming worldwide declines in abundance and diversity (Conrad et al. 2006; Brooks et al 2012; Potts et al. 2010; Hallman et al. 2017). While special attention has focused on declines of both native and managed bee pollinators (Potts et al. 2010; Gallai et al. 2009; Biesmeijer et al. 2006; National Research Council 2007), declines have also been found in a range of other insect taxa, including moths (Conrad et al. 2006; Fox 2013), butterflies (Maes and Van Dyke 2001; Melero et al. 2016; Van Swaay et al. 2010), scarab beetles (Brooks et al. 2012), and flies (Biesmeijer et al. 2006). Further studies find overall declines in insect abundances across large areas, including an alarming 75% decline in abundance of winged insect species across Germany over the past 27 years (Hallman et al. 2017) and an estimated loss of 78-98% of arthropods in Puerto Rican rain forests in just under four decades (Lister and Garcia 2018).

While the consensus is clear that insects are declining globally, the drivers of insect loss remain uncertain, and many notable anthropogenic culprits have been implicated, including pesticides (Goulson et al. 2015; Gill et al. 2012), increased vectoring of pathogens (Cameron et al. 2011; Meeus et al. 2011; Otterstatter et al. 2008; Potts et al. 2010), land use change (Weiner et al. 2014; Winfree et al. 2011), and climate change (Wallisdevries et al. 2006; Giannini et al. 2012; Memmott et al. 2007). Another potential anthropogenic driver of insect declines that has received recent attention is air pollution, particularly tropospheric oxidants, which are highly

reactive chemicals. Tropospheric oxidants may interfere with olfactory cues (Pinto et al. 2010; Jamieson et al. 2017; Jurgens et al. 2017) that insects use to locate both mates and food sources.

I. Olfaction in insects: identifying and locating host plants and mates

Synomones, Kairomones, and Pheromones

Olfaction is so critical to the survival and reproduction of many insect species that olfactory reception has been tightly conserved across hundreds of millions of years, with species from taxonomic groups as diverse as Lepidoptera and Diptera sharing a key functional olfactory reception gene (Jones et al. 2005). Olfactory signals, or semiochemicals, can be classified by their use to sender and receiver organisms: synomones, kairomones, and pheromones. Synomones benefit both the sender and receiver organisms, as in the case of mutualist plant-pollinator signals, where plants produce signals that insects can detect to the mutual benefit of pollinators, which benefit by feeding on floral rewards, and plants, which benefit when pollinators vector their genetic material to conspecifics (Tan et al. 2000). Kairomones benefit the organism that receives a call but not the one that sends it, such as when a herbivore follows a floral scent to a flower (Theis 2006). Pheromones, whether aggregate (Bell et al. 1972) or sexual (Karlson and Luscher 1959), facilitate intraspecific communication.

Floral scents as synomones and kairomones

Plants attract pollinators by advertising their rewards using floral signals, including both visual cues, such as color (Giurfa and Lehrer 2001; Spaethe et al. 2001), shape (Gould 1985), and patterned markings (Penny et al. 1983; Hansen et al. 2012), and olfactory cues (i.e., floral scents). Floral scents can play a particularly important role in pollinator attraction over long-distances, as floral plumes can move downwind of flowers. Floral scents are comprised of plant

secondary metabolites that are secreted from floral or pollen tissue (e.g. Effmer et al. 2005), potentially from specialized cells on the flower called osmophores (Vogel 1962), from floral stomata (Leins 2000), or from glandular trichomes (Kolosova et al. 2001). Recent findings report the active transport of VOCs from plant tissue (Adebesin et al. 2017). Once emitted from the plant, the high volatility of these compounds leads to their quick vaporization, and blends of floral volatiles move downwind, creating a scent trail that pollinators can encounter and follow to the host flower (Metcalf 1987; McFrederick et al. 2008; Dobson et al. 2000; Dudareva et al. 2004) over long distances (e.g., Dressler 1982). Pollinators can detect and recognize the complex blends of volatiles that make up a full floral plume by assessing the concentration of compounds, the specific volatiles present, and combinations of volatiles, potentially including ratios of volatiles within a plume (Wright et al. 2005; Raguso et al. 2008; Wright and Thomson 2005; Bruce et al. 2005)**.** Alternatively, more specialized pollinators, which collect pollen from just a few related plant taxa, may identify host plants using a few or even just one taxonomically distinctive floral volatile compound (Brandt et al. 2016; Dotterl and Vereeken 2010; Milet-Pinheiro et al. 2013; Schaffler et al. 2017).

While plants benefit if floral scents are used as synomones and attract pollinators, they likewise suffer if scents are used as kairomones, as in the case when herbivorous insects detect and use floral signals to locate host plants. Herbivores may eavesdrop on floral volatiles that have coevolved to attract pollinators and use these cues to locate host plants in the same manner that pollinators can (Theis 2006). Plants are thus under pressure to maximize signals that attract pollinators, while at the same time minimizing signals that are attractive to eavesdropping herbivores (Kessler, 2009). While producing such a scent is a moving target for plants, a pathway to this outcome is possible when herbivores and pollinators use different components of the same floral plume (Adler et al. 2009), as in the case where the same floral volatile that attracts a

pollinator also deters herbivores (Kessler et al. 2019). The composition of floral plumes is critical to determining whether a plume attracts pollinators or herbivores or deters herbivores, and thus alterations to the composition of a floral plume could disrupt these interactions.

Sex pheromones

For organisms that reproduce sexually, locating a partner can prove a significant obstacle to mating. To overcome this barrier, organisms across the animal kingdom (Wyat 2014) have employed sex pheromones, which are olfactory signals that broadcast the location of a potential mate. In order to be an effective sex pheromone, a pheromone plume must travel some distance from the calling organism and must be a species-specific identifier to the caller's conspecific. Recognizing pheromone plumes of a conspecific is not always simple, as in the case of the organisms best studied for sex pheromones --moths--where sex pheromone plumes can be remarkably similar in composition even across highly different taxonomic groups (Allison and Carde 2016). Male moths searching for females must distinguish between pheromone plumes based on the exact composition of a plume, using the presence or absence of specific compounds, their abundance, and the ratios of compounds in the pheromone plume (Sakuri et al. 2011; Roelofs et al. 1969; Roelofs et al. 1974). In many known cases, sex pheromone blends are highly specific, and even small deviations in composition, including small changes in the ratio of blend components, can cause pheromone plumes to either lose their attractiveness, or even become actively deterrant, to mate-searching organisms, (Hillier and Baker 2016). Any alterations to the composition of the pheromone plume could thus profoundly interfere with its function as a sex pheromone.

<u>Insect detection and processing of olfactory cues</u>

How do insects detect and process olfactory signals? The primary site of insect olfactory reception is the antenna. As insects filter air through their antenna, odorants are forced into small pores in the otherwise hard cuticle of the antenna. Odorants are trapped in these pores, and move through dendritic tubules from the pore into the lymph of specialized cells or groups of cells shaped in a hair-like structure termed the sensillum. Odors move through the lymph and bind to chemically specific carrier proteins which deliver odors to dendritic branches of olfactory receptors (ORs), with typically 2-4 ORs per sensillum (De Bruyne and Baker 2008; Steinbrecht 1997). ORs only bind with some compounds, and ORs are differentially specific. For the model pollinating moth *Manduca sexta*, there are roughly three different levels of olfactory receptor specificity for floral volatiles, with the most specific group of ORs responding to only one or two chemical compounds. These type of high-specificity ORs are common across several insect species for sex pheromone reception (Kaissling et al 1989; Nakagawa et al 2005; Kurtovic et al 2007). Specialist insects may also have highly specialized ORs that detect unusual compounds that are taxonomically specific to the specialist insect's few host plants. (Brandt et al. 2016). In addition to highly specific ORs, *M. sexta* and other insects also have a second population of ORs that respond to several odorants of the same chemical type (e.g. aromatics) and a third population of ORs that respond to a large range of compounds from different chemical classes (e.g., aromatics, terpenoids etc.) (Shields and Hildebrand 2001).

When an appropriate chemical compound binds to an olfactory receptor, the nerve cell housing the receptor depolarizes, firing off an electrical impulse (Callahan 1975; Vogt and Riddiford 1981; Krieger et al. 1997; Steinbrecht 1997; Riffell et al. 2013). Electrical impulses are transmitted via axons from the OR to glomeruli, a cluster of nerve endings which are housed in the insect's antennal lobe, the site of first-order olfactory processing in the insect's brain (Anton and Homberg, 1999; Hansson and Anton 2000). Both the number and type of olfactory

receptors in the antenna, and the way that these olfactory receptors innervate different glomeruli, create a unique activation pattern in the antennal lobe correlating to a specific sex pheromone or floral plume. These highly specific activation patterns enable an insect to identify either the pheromone plume of a potential mate or the floral plume of a potential host plant based on the concentration and composition of the plumes (Hansson et al. 2003; Carlsson and Hansson 2006). In this way, insects can pick out their host-specific olfactory signal even in backgrounds full of other olfactory inputs (Riffel et al. 2015).

II. Elevated tropospheric oxidants: formation and threat to insect olfactory cues

Anthropogenically driven formation of tropospheric ozone

Both floral scent and sex pheromone cues may be disrupted by elevated tropospheric oxidants, which can react with olfactory signals during transmission through the atmosphere. There are three major species of oxidant in the troposphere: nitrate radical, hydroxyl radical, and ozone. Nitrate radical, formed by oxidation of NO_2 by ozone, is the primary night-time oxidant of VOCs in the troposphere (Monks 2005), although ozone is also a major nighttime oxidant (Yáñez-Serrano et al 2018). Hydroxyl radical is the dominant day-time oxidant, but ozone is present both during the day, when it peaks, and during the evening when hydroxyl radical is no longer formed. Atmospheric lifetime analysis for monoterpenes finds that as a rule, many compounds have a shorter lifetime with respect to hydroxyl radical than the other two oxidants. However, this rule does have prominent exceptions: the common floral volatile linalool has a lifetime of 55 minutes with respect to ozone, compared to 52minutes with respect to hydroxyl radical, and α-Terpinene, another common floral volatile, has a lifetime of 1min with respect to zoone, and a lifetime of 23 minutes with respect to hydroxyl radical (Atkinson and Arey 2003) Although ozone can enter the troposphere from the stratosphere, it is primarily formed in the troposphere

through photochemical reactions (Young et al. 2013). Ozone is a secondary pollutant (Young et al. 2013) and is a major component of smog (Haagen-Smit 1952). Both hydrocarbons and nitrogen oxides (NO_x), which are emitted during fossil fuel combustion, are precursors to ozone formation (Atkinson et al. 1992; Khasibhatla 1993; Jacob 1999). As a secondary pollutant, ozone is primarily formed in the troposphere through photolysis of nitrogen dioxide

$$NO_2 + h\nu \rightarrow NO + O(^3P) \text{ , } \lambda<400 \text{ nm}$$

$$O(^3P) + O_2 \rightarrow O_3$$

Because tropospheric ozone is largely produced from sunlight (300-320 nm) induced photolysis of nitrogen dioxide (NO_2), it has a strong diel cycle, peaking during the afternoon and diminishing during the night. (Jeagle et al. 1998). Unlike hydroxyl radical concentration which directly follows the abundance of sunlight and declines to zero overnight, ozone can linger throughout the evening and is still an important nighttime oxidant (Yáñez-Serrano et al 2018)

Because fossil fuel combustion forms the precursors for ozone formation, the tropospheric load of ozone and other oxidants has increased following the industrial revolution (Vingarzan 2004; Cooper et al. 2013; Naik et al. 2013; Spivakovsky et al. 2000). For North America, the current tropospheric load of ozone for a given year is ~30% higher than in pre-industrial times (Young et al. 2013), with some predictions that ozone will increase by as much as 120% by 2050 for mid-latitudes in July (Brausser et al. 2001). In North America ozone levels can peak during sunny summer days outside of cities, and ozone concentration levels during summertime ozone events in these regions can exceed 120 ppbv (Fiore et al. 2002), a concentration nearly 4-6 times the average North American midlatitude concentration of 20-45 ppbv (Vingarzan 2004). Especially pertinent to agriculture, one of ozone's chemical precursors, peroxyacyl nitrate (PAN) has a long lifetime in the middle and upper troposphere (Liu et al. 1987; Moxim et al. 1996). Given its long lifetime in cold temperatures (several months at 250K)

(Jacobs 1999), PAN can travel great distances through the atmosphere before decomposing to release NO_x and, subsequently, ozone in rural and agricultural areas far from a pollutant source (Van Dingen et al. 2009; Schnell et al. 2009; Cooper et al. 2013). PAN can transport NO_x, which is produced by rapidly increasing fossil fuel combustion in the emerging economies of China and India, from these countries to North America. Thus, background concentrations of tropospheric ozone have been, and are likely to continue to rise for the foreseeable future in North America (The Royal Society 2008; RoTAP 2012).

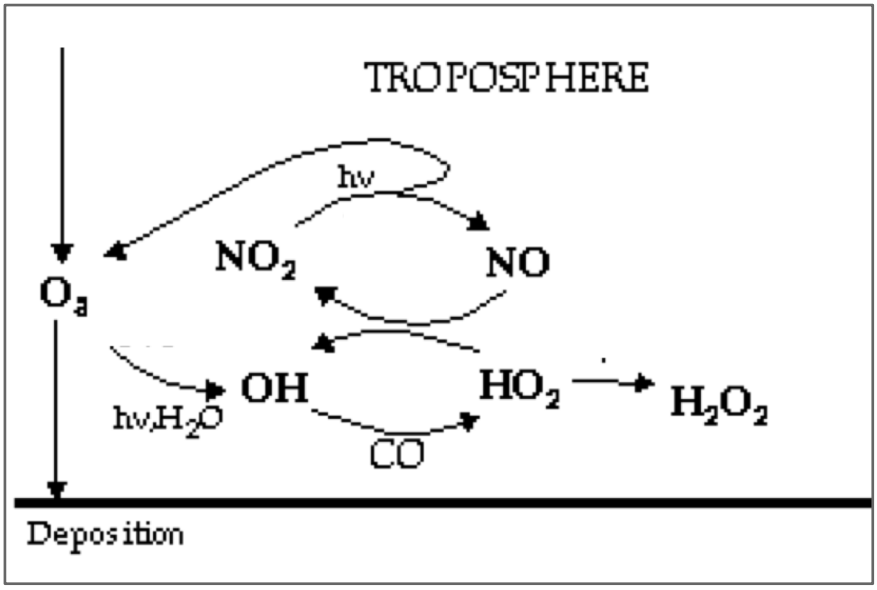

Figure 1. Ozone formation and relations to nitrogen dioxide and hydroxyl radical. Modified from Jacobs 199

Impacts of ozone on plants, insects, and insect olfactory cue use

Impacts of ozone on plants

Elevated concentrations of ozone in rural areas have led to much concern and study on the effects that oxidants, particularly ozone, may have on plant health and agricultural production (Rich 1964; Heath 1980; Krupa and Manning 1988; Sanderman et al. 1998; Morgan et al. 2003; Ashmore 2005; Fiscus 2005; Feng et al. 2008; Van Digen et al. 2009; Long & Naidu 2002).

Ozone enters plants through their stomata; once inside, ozone reacts to form a cascade of secondary oxidants (Heath 2008; Sandermann 2008; Fuhrer 2009) that together can react with lipids, amino acids, and DNA (Heath 2008; Sandermann 2008; Fuhrer 2009). Plants manifest this oxidative stress in a range of responses, including leaf stippling, flecking, bronzing, reddening, chlorosis, or senescence, and decreased plant growth (Rich 1964; Heath 1980; Krupa and Manning 1988; Sanderman et al. 1998; Morgan et al. 2003; Ashmore 2005; Fiscus 2005; Feng et al. 2008). Ozone-exposure also impacts flowers: it induces floral senescence, decreases the number of flowers (Franzaring et al. 2000; Gimeno et al. 2004; Chappelka, A. H. 2002; Black et al. 2000), and may alter floral scent production (Iriti and Faoro 2009; Vuorinen et al. 2004; Khaling et al. 2016). Ozone exposure can also impact floral rewards, decreasing pollen viability (Pasqualini et al. 2011) and potentially altering nectar volume and sugar allocation (Witton 2016; Stabler 2016). If ozone-exposure alters plants' production of floral signals and rewards, plant-pollinator interactions may be altered in polluted environments.

Impacts of ozone on insect and insect antenna

In a similar manner to which ozone reacts with plant tissue and harms plants, ozone may also react with insect tissue and harm insects. The proposed mechanism for ozone damage to insect tissue is peroxidation of lipids, though one study found fumigating red flour beetles with ozone did not peroxidize lipids as expected but did decrease beetle movement and lead to some mortality (Holstrump et al. 2011). Current understanding of ozone's effect on insect health is limited and has primarily focused on the behavioral response of an insect to ozone fumigation (Hay 1977). When used as a fumigant, ozone is produced at unnaturally high concentrations, in the range of 25-60 ppm (Hay 1977 and citations therein). At this fumigation level, ozone can cause insect mortality: ozone fumigation induced mortality in two species of rice weevils

(Yoshida 1975), three species of Diptera (Levy et al. 1974; Levy et al. 1972), as well as the flour beetle *Tribolium castaneum* and meal moth *Plodia interpunctella* (Kells et al. 2001). When not causing mortality, ozone fumigation induced sub-lethal side effects, including interference with mitosis in grasshoppers (Fetner et al. 1963) and reduced walking rates in maize weevils (Sousa et al. 2012). The impact of ozone on insect antennae and perception of olfactory signals has been less well-studied: there is possibly only one study that attempted to test the effects of ozone on a pollinator's olfactory perception (Dötterl et al. 2016). This study presented a floral volatile in 1000 ppbv of ozone and found a decrease in honeybees' antennal response to the odor presented in ozone vs. air (Dötterl et al. 2016). The researchers argue that ozone and the odorant would not have had sufficient time to react, so the insect's decreased antennal response (assessed via electroantennogram) was driven by the presence of ozone rather than a decrease in concentration of the odor.

Impacts of air pollution on insect olfactory cue recognition

In addition to altering the production and potentially detection of important insect olfactory signals, atmospheric pollution may react with and deteriorate olfactory signals as they travel downwind. Atmospheric oxidants such as ozone could react with the chemical compounds that make up complex floral or sex pheromone plumes. The majority of floral volatiles examined thus far are reactive with ozone (Atkinson and Arey 2003; Baker et al. 2002), and the only report of sex pheromones exposed to ozone found that the majority of pheromone compounds were also reactive with ozone (Klumpp et al. 2000). Oxidant reaction with floral or pheromone plumes during transmission could have two impacts on olfactory signals. Firstly, oxidants react with compounds and thus reduce the distance that those signals travel intact through the atmosphere. Atmospheric chemistry models find that tropospheric oxidants at peaks of 80 and 120 ppbv can

reduce the range of specific common floral volatiles, decreasing the abundance of some of these compounds beneath insect detection thresholds at 800m or less (McFrederick et al. 2007; Fuentes et al. 2016). These results are further supported in an empirical study that found that at a distance of 4.5m, the overall volume of floral volatiles was reduced by 25–30% after mixing with 120 ppbv ozone (Farre-Armengol et al. 2016). By diminishing the reach of these signals, air pollutants can reduce their effectiveness as signals.

Secondly, air pollutants may also induce compositional changes to floral plumes that render the plumes less attractive to foraging insects (Farre-Armengol et al. 2016; Li et a 2016). In both floral and sex pheromone plumes, atmospheric oxidants are likely to react with some, but not all, components of the plume, thus altering the ratios of compounds in the plume. Both blends and ratios of compounds are important for conspecific recognition of sex pheromone plumes (Allison and Carde 2016). Likewise, blends, and potentially ratios of compounds in a blend, can also be important for insect recognition of floral plumes (Bruce et al. 2005; Bruce and Picketts 2011) or of mates (Allison and Carde 2016). Moreover, the plume acquires secondary compounds as ozone reacts with primary floral volatiles and creates break-down compounds which are added to the plume (Li et al. 2016; McFrederick et al. 2007; Lee et al. 2006). For floral plumes, these secondary compounds may be long-lasting volatiles similar in structure and potentially function to other floral volatiles: one such compound is 6-methyl-5-hepten-2-one, which is formed from oxidation of the common floral volatile linalool but also serves as an important floral attractant to pollinators (Fruekilde et al. 1998; Tollsten & Bergström 1993). However, secondary compounds also include chemicals that are highly dissimilar to the parent compounds, including acetone, formaldehyde, and carbon monoxide (McFrederick et al. 2007; Lee at al 2006). As a result of these ozone-induced alterations, the plume may become less attractive to foraging insects (Fuentes et al. 2013; Farre-armengol et al. 2016; Li et al. 2016).

Furthermore, while no studies have investigated the behavioral response of insects to ozone-altered pheromone plumes, at least one field study finds that elevated ozone can diminish aggregation behavior in aphids that use pheromone aggregates (Mondor et al. 2004), indicating that pheromone plumes, such as floral plumes, may be rendered less attractive or even unattractive to their intended recipients.

III. Insect olfactory cue learning and application to pollution-altered olfactory cues

Ozone exposure to plumes induces both a temporal and spatial variation in signals that are critical to pollinators. Ozone can decrease the distance that innately attractive or associatively learned floral scents travel intact. Also, because ozone has a diel cycle as well as seasonal and event-related peaks, plumes that are reactive with ozone can change rapidly both day-to-day and within a day, inducing temporal as well as spatial variability in floral plumes.

However, insects may have an evolutionarily formed coping mechanism to deal with variability in olfactory cues: olfactory learning. Olfactory cue learning via association of reward with an unknown odor has been demonstrated in many insect species. Olfactory learning may enable insects to reestablish attraction to floral scents that have been altered by atmospheric oxidants--particularly to those insects that have incorporated olfactory learning as part of their evolutionarily-formed foraging strategy. This includes both herbivore and pollinator generalists: these insects may have an innate attraction to some olfactory cues but hone their preference for host-plants based on experience. These insects can form an association between the scent of a given host plant and the quality and quantity of the food rewards that the plant provides. Associations between odor and reward may be positive, if the reward quality is sufficiently high, or negative, if the rewards are low (Weigmann et al. 2003) or toxic (Stevenson et al. 2017). In pollinators, olfactory cue learning enforces honesty in floral signals (Wright and Schiestl 2009;

Knauer and Schiestl 2014), as pollinators foraging on a flower with sufficiently high quality rewards form a positive association and can then selectively choose to forage on plants presenting the same signal. This benefits plants by maximizing the transfer of pollen to conspecifics and benefits pollinators by maximizing their efficiency for locating plants (Wilson and Stine 1996; Raine and Chittka 2006). A plethora of lab studies provide testament to pollinators' excellent ability to learn olfactory cues (Wright et al. 2009; Deisig 2001; Cunningham 2004). Furthermore, while olfactory cue learning has not been as well examined in the case of herbivores as in the case of pollinators, several studies find that herbivores too can associate plant odors with rewards and use this information to select the most rewarding or least harmful host-plant available (Simoes et al. 2011; Costa, & Reeve 2011; Papaj and Prokopy 1989).

Olfactory learning has several limitations, however. Not all insects may have the neurological ability to learn olfactory cues. Notably, specialist insects may not have the same ability as generalist insects to learn olfactory cues. In contrast to generalist insects, which have incorporated learning into their foraging strategy, specialist insects, which may rely on a few uncommon and taxonomically specific compounds to locate their host plants, may have no need to learn new olfactory cues to select favored host plants. Emerging studies on learning in specialist insects report only limited abilities to learn olfactory cues (Milet-Pinheiro et al. 2012; Burger et al. 2010). Furthermore, the most highly specialized olfactory cues used by insects are likely to be sex pheromones, and learning is not likely to assist insects in restablishing atttraction to pollution-altered sex pheromones.

Learning could be achieved in sex-pheromone mediated mate location if an insect followed a generalized pheromone blend intially, and honed preference for the pheromone plume of its conspecific based on its encounters with non-conspecifics, less desireable conspecifics, and

hihgly desireable conspecifics. However, current studies on sex pheromones in moths find that sex pheromones are species specific to naive insects, and even small changes to the composition of a sex pheromone plume dictate whether it will be perceived as either key pheromone attractant or an active deterrent to a mate-searching insect (Hillier and Baker 2016). Indeed, the strong behavioral response to small changes in pheromone plume composition are taken as an argument that moths have evolved a means of stringently detecting the plume of their conspecifics while avoiding similar plumes from congener or other closely related species (Hillier and Baker 2016).

Learnign sex pheromones might also be achieved if there is variation in sex pheromone blends within a species, and mates can, based on their mating experiences, select pheromone blends that lead to the best quality conspecific mate. However, this scenario is unlikely as there is no obvious way for an insect to associate a sex pheromone blend with success or failure, as the outcome of a mating, offspring and their fitness, is temporally decoupled from the mating act.

In polluted environments, learning olfactory cues may play an expanded role in host location in highly polluted atmospheres by enabling insects to recognize initially-unattractive ozone-altered olfactory cues. If olfactory cue learning can be used as a strategy to recognize ozone-altered plumes and mitigate the potential negative impacts of ozone, then insects using a specialized foraging strategy, which does not require the development of strong odor-learning abilities, may be at higher risk than their generalist counterparts, and all sex-pheromone mediate mate location may be at risk.

Even in the case of generalist foragers, it is unclear if evolved learning abilities will translate to an ability to learn ozone-altered scents. In order to learn ozone-altered plumes, insects would have to perform a more complex learning mechanism than is necessary to cope with natural variation in plumes in an unpolluted landscape. Because floral plumes only become altered by ozone as they move away from the floral source, they are decoupled from the nectar or

pollen rewards offered at the flower. Thus, in order to learn ozone-altered plumes, pollinators would have to form an association between ozone-altered plumes that are temporally and spatially separated from the floral rewards. There are at least three learning mechanisms that would enable insects to learn ozone-altered plumes in a landscape. Insects could learn unreacted (i.e. ozone-resistant) compounds in the plume at the rewarding plant (Reinhard et al. 2010) and then recognize them later in the ozone-altered plumes. Insects could also generalize between compounds of similar structure (Daly et al. 2001) in the ozone-exposed and pure plumes. Finally, insects may be able to link the ozone-altered plume to the unaltered plume at the flower, as linking of two sequential odors leading to a reward has been achieved by honeybees (Hussaini et al. 2007). Currently, no studies have investigated the ability of foraging insects to learn ozone-altered plumes that they do not initially find attractive.

SIGNIFICANCE

Investigating the impacts of elevated tropospheric oxidants on plant-insect interactions can provide important information relevant to insect and pollination conservation and may offer insights into the resiliency of coevolved systems under anthropogenic perturbations.

Air-pollution induced disruptions to floral scents that mediate plant-pollinator interactions could have profound detrimental impacts on pollination. This threat is further compounded, as regions with the highest current and projected air pollution levels, those countries at the peak of their industrialization period, are also countries where increases in human population create a demand for increasing agricultural production (Godfray et al. 2009). Thus, the impact of air pollution is not only a matter of current concern but one that may increase in industrializing nations over time.

While managed bees may be less foraging efficient in polluted environments, the placement of their colonies directly in and around crops may make them less vulnerable to some of the impacts of elevated tropospheric oxidants, as they may be placed close enough to target plants to rely on visual cues to find them. Wild pollinators, on the other hand, may emerge as adults far from either their preferred host plants or from agricultural plots. In the absence of visual cues, and with only ozone-degraded olfactory cues, emerging adult wild pollinators may be less efficient at locating host plants or might never locate agricultural crops that would otherwise benefit from wild pollination. Ozonation of floral olfactory scents could prove especially pernicious in the case of specialist insects that rely on a few token compounds to locate specific host plants; in ozone-enriched environments, those olfactory cues may be eroded, and specialist insects emerging at some distance from their host plants may be left without visual or olfactory cues to direct their search. For agricultural systems that utilize wild pollinators (Winfree 2008; Goulson 2003), including specialist wild pollinators (Tepedino et al. 1981), elevated air pollution could jeopardize productivity.

Tropospheric oxidants may also drive a sudden shift in plant-insect interactions that have developed over evolutionary timescales, affecting both agricultural and managed ecosystems in ways currently unknown. By altering floral signals, tropospheric oxidants may drive change in plant-insect interactions and thus in plant success and community structuring. Pollinator choice may be altered in polluted environments: for example, pollinators may be more likely to encounter primarily unreactive floral scents or might potentially prefer flower species that increase rather than maintain their production of nectar under oxidative stress. While alterations to the emitted floral plume by atmospheric pollution are not likely to enhance its attractiveness to foraging insects, plume alterations can affect organisms differently depending on which cues a particular organism uses. In cases where pollinators and herbivores utilize different floral cues,

ozonation may cause plumes to be less attractive to pollinators relative to herbivores or vice-versa. Oxidant stressed plants are more attractive to some herbivores (Vourinen et al. 2004; Holopainen et al. 2011) but have not been found to be more attractive to mutualist insects (Khaling et al. 2016; Vuorinen et al. 2004). Thus, the attractiveness of plants to herbivores relative to pollinators may be shifted under increasingly polluted atmospheres, potentially altering individual plant species' success and eventually causing changes in community dynamics.

Finally, recent studies report alarming declines in insect abundances worldwide (Sanchez-Bayo and Wyckhuys et al. 2019; Lister and Garcia 2018; Hallman et al. 2017). Atmospheric pollution, which can interfere with the olfactory cues that insects use to locate host plants and especially those used to locate mates, could be a factor contributing to the global decline of these important organisms. Further research is needed to assess whether the pernicious impacts of air pollution on insect cues could impact foraging and mate-locating efficiency and preferences.

Citations

Adebesin, F., Widhalm, J. R., Boachon, B., Lefèvre, F., Pierman, B., Lynch, J. H., & Porter, J. A. (2017). Emission of volatile organic compounds from petunia flowers is facilitated by an ABC transporter. *Science*, *356*(6345), 1386-1388.

Ahmad, S. (1995). Oxidative stress from environmental pollutants. *Archives of insect biochemistry and physiology*, *29*(2), 135-157

Allison, J. D., & CardŽ, R. T. (2016). *Pheromone communication in moths: evolution, behavior, and application*. Univ of California Press.

Alstad, D. N., Edmunds Jr, G. F., & Weinstein, L. H. (1982). Effects of air pollutants on insect populations. *Annual Review of Entomology*, *27*(1), 369-384.

Alvarado, A., Arey, J., and Atkinson, R., 1998: Kinetics of the gas-phase reactions of OH and NO3 radicals and O3 with the monoterpene reaction products pinonaldehyde, caronaldehyde, and sabinaketone, J. Atmos. Chem. 3 1, 281–297

Andrews, E. S., Theis, N., & Adler, L. S. (2007). Pollinator and herbivore attraction to Cucurbita floral volatiles. *Journal of chemical ecology*, *33*(9), 1682-1691.

Anton, S., & Homberg, U. (1999). Antennal lobe structure. In Insect olfaction (pp. 97-124). Springer Berlin Heidelberg.

Ashmore, M. R. (2005). Assessing the future global impacts of ozone on vegetation. *Plant, Cell & Environment*, *28*(8), 949-964.

Atkinson, R., & Arey, J. (2003). Gas-phase tropospheric chemistry of biogenic volatile organic compounds: a review. *Atmospheric Environment*, *37*, 197-219.

Baker J., Arey, J., & Atkinson, R. (2004). Kinetics of the gas-phase reactions of OH radicals, NO3 radicals and O3 with three C7-carbonyls formed from the atmospheric reactions of myrcene, ocimene and terpinolene. *Journal of atmospheric chemistry*, *48*(3), 241-260.distribution and trends of tropospheric ozone: An observation-based review. *Elementa*, *2*.

Barnes, J. D., & Wellburn, A. R. (1998). Air pollutant combinations. *Responses of plant metabolism to air pollution and global change. Leiden: The Netherlands: Backhuys Publishing, pp147–164*.

Beauchamp, J., Wisthaler, A., Hansel, A., Kleist, E., Miebach, M., NIINEMETS, Ü., ... & WILDT, J. (2005). Ozone induced emissions of biogenic VOC from tobacco: relationships between ozone uptake and emission of LOX products. *Plant, Cell & Environment*, *28*(10), 1334-1343.

Bergweiler, C. J., & Manning, W. J. (1999). Inhibition of flowering and reproductive success in spreading dogbane (Apocynum androsaemifolium) by exposure to ambient ozone. *Environmental Pollution*, *105*(3), 333-339.

Bell, W. J., Parsons, C., & Martinko, E. A. (1972). Cockroach aggregation pheromones: analysis of aggregation tendency and species specificity (Orthoptera: Blattidae). *Journal of the Kansas Entomological Society*, 414-421.

Beyaert, I., & Hilker, M. (2014). Plant odour plumes as mediators of plant–insect interactions. *Biological Reviews*, *89*(1), 68-81.

Biesmeijer, J. C., Roberts, S. P., Reemer, M., Ohlemüller, R., Edwards, M., Peeters, T., ... & Settele, J. (2006). Parallel declines in pollinators and insect-pollinated plants in Britain and the Netherlands. *Science*, *313*(5785), 351-354.

Black VJ, Black CR, Roberts JA, Stewart CA (2000) Impact of ozone on the reproductive development of plants. New Phytologist, 147, 421–447.

Black VJ, Stewart CA, Roberts JA, Black CR (2007) Ozone affects gas exchange, growth and reproductive development in Brassica campestris (Wisconsin Fast Plants). New Phytologist, 176, 150–163.

Blande, J. D., Holopainen, J. K., & Li, T. (2010). Air pollution impedes plant-to-plant communication by volatiles. *Ecology letters*, *13*(9), 1172-1181.

Bruasseur, G. P., Muller, J. F., Tie, X., & Horowitz, L. (2001). Tropospheric ozone and climate: past, present and future. *Present and Future of Modeling Global Environmental Change: Toward Integrated Modeling. TERRAPUB*, 63-75.

Brandt, K., Dötterl, S., Francke, W., Ayasse, M., & Milet-Pinheiro, P. (2016). Flower Visitors of Campanula: Are Oligoleges More Sensitive to Host-Specific Floral Scents Than Polyleges?. *Journal of Chemical Ecology*, 1-9.

Brodschneider, R., & Crailsheim, K. (2010). Nutrition and health in honey bees. *Apidologie*, *41*(3), 278-294.

Brooks, D. R., Bater, J. E., Clark, S. J., Monteith, D. T., Andrews, C., Corbett, S. J., ... & Chapman, J. W. (2012). Large carabid beetle declines in a United Kingdom monitoring network increases evidence for a widespread loss in insect biodiversity. *Journal of Applied Ecology*, *49*(5), 1009-1019.

Burger, Hannah, Stefan Dötterl, and Manfred Ayasse. "Host-plant finding and recognition by visual and olfactory floral cues in an oligolectic bee." *Functional Ecology* 24.6 (2010): 1234-1240.

Burger H, Do¨tterl S, Ayasse M (2010b) Host plant finding and recognition by visual and olfactory floral cues in an oligolectic bee. Funct Ecol 24:1234 1240

Callahan, P. S. (1975). Insect antennae with special reference to the mechanism of scent detection and the evolution of the sensilla. International Journal of Insect Morphology and Embryology, 4(5), 381-430.

Cameron, S. A., Lozier, J. D., Strange, J. P., Koch, J. B., Cordes, N., Solter, L. F., & Griswold, T. L. (2011). Patterns of widespread decline in North American bumble bees. *Proceedings of the National Academy of Sciences*, *108*(2), 662-667.

Carlsson, M. A., Galizia, C. G., & Hansson, B. S. (2002). Spatial representation of odours in the antennal lobe of the moth Spodoptera littoralis (Lepidoptera: Noctuidae). *Chemical senses*, *27*(3), 231-244.

Chittka, L., & Raine, N. E. (2006). Recognition of flowers by pollinators. *Current opinion in plant biology*, *9*(4), 428-435.

Comtois, P., & Schemenauer, R. S. (1991). Tree pollen viability in areas subject to high pollutant deposition. *Aerobiologia*, *7*(2), 144-151.

Collett, T. S., & Collett, M. (2002). Memory use in insect visual navigation. *Nature Reviews Neuroscience*, *3*(7), 542-552.

Cooper, O. R., Parrish, D. D., Ziemke, J., Balashov, N. V., Cupeiro, M., Galbally, I. E., ... & Naik, V. (2014). Global

Cook, S. M., Bartlet, E., Murray, D. A., & Williams, I. H. (2002). The role of pollen odour in the attraction of pollen beetles to oilseed rape flowers. *Entomologia experimentalis et applicata*, *104*(1), 43-50.

Conrad K.F Warren, M. S., Fox, R., Parsons, M. S., & Woiwod, I. P. (2006). Rapid declines of common, widespread British moths provide evidence of an insect biodiversity crisis. *Biological conservation*, *132*(3), 279-291.

Costa, A., & Reeve, J. D. (2011). Olfactory experience modifies semiochemical responses in a bark beetle predator. *Journal of chemical ecology*, *37*(11), 1166-1176.

Cruden, R. W. (1972). Pollination biology of Nemophila menziesii (Hydrophyllaceae) with comments on the evolution of oligolectic bees. *Evolution*, 373-389.

Cunningham, S.A. (1995). Ecological constraints on fruit initiation by Calyptrogyne ghiesbreghtiana (Arecaceae): floral herbivory, pollen availability, and visitation by pollinating bats. Am. J. Bot., 82, 1527–1536.

Cunningham, J. P., Moore, C. J., Zalucki, M. P., & West, S. A. (2004). Learning, odour preference and flower foraging in moths. *Journal of Experimental Biology*, *207*(1), 87-94.

Daly, K. C., Christensen, T. A., Lei, H., Smith, B. H., & Hildebrand, J. G. (2004). Learning modulates the ensemble representations for odors in primary olfactory networks. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(28), 10476-10481.

Daly, K. C., Durtschi, M. L., & Smith, B. H. (2001). Olfactory-based discrimination learning in the moth, Manduca sexta. *Journal of Insect Physiology*, *47*(4), 375-384.

David, C. T., Kennedy, J. S., & Ludlow, A. R. (1983). Finding of a sex pheromone source by gypsy moths released in the field. *Nature*, *303*(5920), 804.

De Bruyne, M., & Baker, T. C. (2008). Odor detection in insects: volatile codes. *Journal of chemical ecology*, *34*(7), 882-897.

Deisig, N., Lachnit, H., Giurfa, M., & Hellstern, F. (2001). Configural olfactory learning in honeybees: negative and positive patterning discrimination. *Learning & Memory*, *8*(2), 70-78.
Dobson, H. E., Bergström, J., Bergström, G., & Groth, I. (1987). Pollen and flower volatiles in two Rosa species. *Phytochemistry*, *26*(12), 3171-3173.
Dobson, H. E. (1994). Floral volatiles in insect biology. *Insect-plant interactions*, *5*, 47-81.
Dobson, H. E., Groth, I., & Bergstrom, G. (1996). Pollen advertisement: chemical contrasts between whole-flower and pollen odors. *American Journal of Botany*, 877-885.
Dobson, H. E., & Bergström, G. (2000). The ecology and evolution of pollen odors. *Plant Systematics and Evolution*, *222*(1-4), 63-87.
Dötterl, S., Füssel, U., Jürgens, A., & Aas, G. (2005). 1, 4-Dimethoxybenzene, a floral scent compound in willows that attracts an oligolectic bee. *Journal of chemical ecology*, *31*(12), 2993-2998.
Dötterl, S., & Schäffler, I. (2007). Flower scent of floral oil-producing Lysimachia punctata as attractant for the oil-bee Macropis fulvipes. *Journal of chemical ecology*, *33*(2), 441-445.
Do¨tterl S, Verecken NJ (2010) The chemical ecology and evolution of bee flower interactions: a review and perspectives. Can J Zool 88:668 697
Dötterl, S., Vater, M., Rupp, T., & Held, A. (2016). Ozone Differentially Affects Perception of Plant Volatiles in Western Honey Bees. *Journal of Chemical Ecology*, *42*(6), 486-489.
Dressler, R. L. (1982). Biology of the orchid bees (Euglossini). *Annual Review of Ecology and Systematics*, *13*(1), 373-394.
Dudareva, N., Pichersky, E., & Gershenzon, J. (2004). Biochemistry of plant volatiles. *Plant physiology*, *135*(4), 1893-1902.
Dukas, R. (2001). Effects of perceived danger on flower choice by bees. *Ecology Letters*, *4*(4), 327-333.
Effmert, Uta, et al. "Volatile composition, emission pattern, and localization of floral scent emission in Mirabilis jalapa (Nyctaginaceae)." *American journal of botany* 92.1 (2005): 2-12.
Faoro, F., & Iriti, M. (2009). Plant cell death and cellular alterations induced by ozone: key studies in Mediterranean conditions. *Environmental Pollution*, *157*(5), 1470-1477.
Fiscus EL, Booker FL, Burkey KO (2005) Crop responses to ozone: uptake, modes of action, carbon assimilation and partitioning. Plant, Cell and Environment, 28, 997–1011.
Feng Z, Kobayashi K, Ainsworth EA (2008) Impact of elevated ozone concentration on growth, physiology, and yield of wheat (Triticum aestivum L.): a meta-analysis. Global Change Biology, 14, 2696–2708.
Fox R. The decline of moths in Great Britain: a review of possible causes Insect Conserv. Divers., 6 (2013), pp. 5-19.
Fuhrer, J. (2009). Ozone risk for crops and pastures in present and future climates. Naturwissenschaften, 96(2), 173-194.
Gallai, N., Salles, J. M., Settele, J., & Vaissière, B. E. (2009). Economic valuation of the vulnerability of world agriculture confronted with pollinator decline. *Ecological economics*, *68*(3), 810-821.
Giannini, T. C., Acosta, A. L., Garófalo, C. A., Saraiva, A. M., Alves-dos-Santos, I., & Imperatriz-Fonseca, V. L. (2012). Pollination services at risk: bee habitats will decrease owing to climate change in Brazil. *Ecological Modelling*, *244*, 127-131.
Gill, R. J., Ramos-Rodriguez, O., & Raine, N. E. (2012). Combined pesticide exposure severely affects individual-and colony-level traits in bees. *Nature*, *491*(7422), 105.

Giurfa, M. & Lehrer, M. (2001). Honeybee vision and floral displays: from detection to close-up recognition. *Cognitive ecology of pollination*, 61-82.
Godfray, H. C. J., Crute, I. R., Haddad, L., Lawrence, D., Muir, J. F., Nisbett, N., ... & Whiteley, R. (2010). The future of the global food system.
Goulson, D. (2003). Conserving wild bees for crop pollination. *Journal of Food Agriculture and Environment*, *1*, 142-144.
Goulson, D., Nicholls, E., Botías, C., & Rotheray, E. L. (2015). Bee declines driven by combined stress from parasites, pesticides, and lack of flowers. *Science*, *347*(6229), 1255957.
Greenfield, M. D. (1981). Moth sex pheromones: an evolutionary perspective. *The Florida Entomologist*, *64*(1), 4-17.
Haagen-Smit, A. J. (1952). Chemistry and physiology of Los Angeles smog. *Industrial & Engineering Chemistry*, *44*(6), 1342-1346.
Hallmann CA, Sorg M, Jongejans E, Siepel H, Hofland N, Schwan H, et al. More than 75 percent decline over 27 years in total flying insect biomass in protected areas. Plos One. 2017;12(10)
Hansen, D. M., Van der Niet, T., & Johnson, S. D. (2012). Floral signposts: testing the significance of visual 'nectar guides' for pollinator behaviour and plant fitness. *Proceedings of the Royal Society of London B: Biological Sciences*, *279*(1729), 634-639.
Hansson, B. S., Carlsson, M. A., & Kalinova, B. (2003). Olfactory activation patterns in the antennal lobe of the sphinx moth, Manduca sexta. *Journal of Comparative Physiology A*, *189*(4), 301-308
Heath, R. L. (1980). Initial events in injury to plants by air pollutants. *Annual review of plant physiology*, *31*(1), 395-431.
Heath, R. L. (1994). Possible mechanisms for the inhibition of photosynthesis by ozone. *Photosynthesis Research*, *39*(3), 439-451.
Hay, J. C. (1977). Bibliography on Arthropoda and air pollution.
Heath, R. L. (1980). Initial events in injury to plants by air pollutants. *Annual review of plant physiology*, *31*(1), 395-431.
Heath, R. L. (1994). Possible mechanisms for the inhibition of photosynthesis by ozone. *Photosynthesis Research*, *39*(3), 439-451.
Heard, D. E., Carpenter, L. J., Creasey, D. J., Hopkins, J. R., Lee, J. D., Lewis, A. C., ... & Emmerson, K. M. (2004). High levels of the hydroxyl radical in the winter urban troposphere. *Geophysical research letters*, *31*(18).
Hillier, N. K., & Baker, T. C. (2016). Pheromones of Heliothine moths. In *Pheromone Communication in Moths-Evolution, Behavior, and Application* (pp. 301-333). University of California Press.
Himanen, S. J., Nerg, A. M., Nissinen, A., Pinto, D. M., Stewart, C. N., Poppy, G. M., & Holopainen, J. K. (2009). Effects of elevated carbon dioxide and ozone on volatile terpenoid emissions and multitrophic communication of transgenic insecticidal oilseed rape (Brassica napus). *New Phytologist*, *181*(1), 174-186.
Holmstrup, M., Sørensen, J. G., Heckmann, L. H., Slotsbo, S., Hansen, P., & Hansen, L. S. (2011). Effects of ozone on gene expression and lipid peroxidation in adults and larvae of the red flour beetle (Tribolium castaneum). *Journal of stored products research*, *47*(4), 378-384.
Hussaini, S. A., Komischke, B., Menzel, R., & Lachnit, H. (2007). Forward and backward second-order Pavlovian conditioning in honeybees. *Learning & Memory*, *14*(10), 678-683.

Iriti, M., & Faoro, F. (2009). Chemical diversity and defence metabolism: how plants cope with pathogens and ozone pollution. *International journal of molecular sciences*, *10*(8), 3371-3399.

Jacob, D. J. (1999). *Introduction to atmospheric chemistry*. Princeton University Press.

Jaeglé, L., Jacob, D. J., Brune, W. H., Tan, D., Faloona, I. C., Weinheimer, A. J., ... & Sachse, G. W. (1998). Sources of HOx and production of ozone in the upper troposphere over the United States. *Geophysical Research Letters*, *25*(10), 1709-1712.

Jones W. D., Nguyen, T. A. T., Kloss, B., Lee, K. J., & Vosshall, L. B. (2005). Functional conservation of an insect odorant receptor gene across 250 million years of evolution. Current Biology, 15(4), R119-R121

Kessler, André, and Rayko Halitschke. Testing the potential for conflicting selection on floral chemical traits by pollinators and herbivores: predictions and case study. *Functional Ecology* 23.5 (2009): 901-912

Kaissling, K. E., Hildebrand, J. G., & Tumlinson, J. H. (1989). Pheromone receptor cells in the male moth Manduca sexta. *Archives of Insect Biochemistry and Physiology*, *10*(4), 273-279.

Kessler, D., Bing, J., Haverkamp, A., & Baldwin, I. T. (2019). The defensive function of a pollinator-attracting floral volatile. *Functional Ecology*.

Klein, A. M., Vaissiere, B. E., Cane, J. H., Steffan-Dewenter, I., Cunningham, S. A., Kremen, C., & Tscharntke, T. (2006). Importance of pollinators in changing landscapes for world crops. *Proceedings of the royal society B: biological sciences*, *274*(1608), 303-313.

Kolosova, N., Sherman, D., Karlson, D., & Dudareva, N. (2001). Cellular and Subcellular Localization ofS-Adenosyl-l-Methionine: Benzoic Acid Carboxyl Methyltransferase, the Enzyme Responsible for Biosynthesis of the Volatile Ester Methylbenzoate in Snapdragon Flowers. *Plant Physiology*, *126*(3), 956-964

Kugler, H. 1970. Blu¨teno¨kologie. Gustav Fischer Verlag, Jena, Germany.

Kunze, J., & Gumbert, A. (2001). The combined effect of color and odor on flower choice behavior of bumble bees in flower mimicry systems. *Behavioral Ecology*, *12*(4), 447-456.

Kurtovic, A., Widmer, A., & Dickson, B. J. (2007). A single class of olfactory neurons mediates behavioural responses to a Drosophila sex pheromone. *Nature*, *446*(7135), 542.

Krieger, J., Mameli, M., & Breer, H. (1997). Elements of the olfactory signaling pathways in insect antennae. *Invertebrate neuroscience*, *3*(2-3), 137-144

Krupa, S. V., & Manning, W. J. (1988). Atmospheric ozone: formation and effects on vegetation. *Environmental Pollution*, *50*(1-2), 101-137.

National Research Council. (2007). *Status of pollinators in North America*. National Academies Press. DOI: https://doi.org/10.17226/11761

Lewis, P. A., Lampman, R. L., & Metcalf, R. L. (1990). Kairomonal attractants for Acalymma vittatum (Coleoptera: Chrysomelidae). *Environmental entomology*, *19*(1), 8-14

Lister, B. C., & Garcia, A. (2018). Climate-driven declines in arthropod abundance restructure a rainforest food web. *Proceedings of the National Academy of Sciences*, *115*(44), E10397-E10406.

Liu, S. C., Trainer, M., Fehsenfeld, F. C., Parrish, D. D., Williams, E. J., Fahey, D. W., ... & Murphy, P. C. (1987). Ozone production in the rural troposphere and the implications for regional and global ozone distributions. *Journal of Geophysical Research: Atmospheres*, *92*(D4), 4191-4207.

Losey, J. E., & Vaughan, M. (2006). The economic value of ecological services provided by insects. *Bioscience*, *56*(4), 311-323.

Maes, H. Van Dyck Butterfly diversity loss in Flanders (north Belgium): Europe's worst case scenario? Biol. Conserv., 99 (2001), pp. 263-276
McFrederick, Q. S., Kathilankal, J. C., & Fuentes, J. D. (2008). Air pollution modifies floral scent trails. *Atmospheric Environment*, *42*(10), 2336-2348.
McFrederick, Q. S., Fuentes, J. D., Roulston, T. A., Kathilankal, J. C., & Lerdau, M. (2009). Effects of air pollution on biogenic volatiles and ecological interactions. *Oecologia*, *160*(3), 411-420.
McGregor, S. E. (1976). *Insect pollination of cultivated crop plants* (Vol. 496). Washington, DC: Agricultural Research Service, US Department of Agriculture.
Meeus, I., Brown, M. J., De Graaf, D. C., & Smagghe, G. U. Y. (2011). Effects of invasive parasites on bumble bee declines. *Conservation Biology*, *25*(4), 662-671.
Melero, C. Stefanescu, J. PinoGeneral declines in Mediterranean butterflies over the last two decades are modulated by species traits Biol. Conserv., 201 (2016), pp. 336-34.
Memmott, J., Craze, P. G., Waser, N. M., & Price, M. V. (2007). Global warming and the disruption of plant–pollinator interactions. *Ecology letters*, *10*(8), 710-717.
Metcalf, R. L., & Metcalf, E. R. (1992). *Plant kairomones in insect ecology and control*. Chapman and Hall Ltd.
Metcalf, R. L., & Kogan, M. (1987). Plant volatiles as insect attractants. *Critical reviews in plant sciences*, *5*(3), 251-301.
Mondor, E. B., Tremblay, M. N., Awmack, C. S., & Lindroth, R. L. (2004). Divergent pheromone-mediated insect behaviour under global atmospheric change. *Global Change Biology*, *10*(10), 1820-1824
Moxim, W. J., Levy, H., & Kasibhatla, P. S. (1996). Simulated global tropospheric PAN: Its transport and impact on NO x. Journal of Geophysical Research: Atmospheres, 101(D7), 12621-12638.
Monks, P. S. (2005). Gas-phase radical chemistry in the troposphere. *Chemical Society Reviews*, *34*(5), 376-395.
Morgan, P. B., Ainsworth, E. A., & Long, S. P. (2003). How does elevated ozone impact soybean? A meta-analysis of photosynthesis, growth and yield. *Plant, Cell & Environment*, *26*(8), 1317-1328.
Naik, V., Voulgarakis, A., Fiore, A. M., Horowitz, L. W., Lamarque, J. F., Lin, M., ... & Cionni, I. (2013). Preindustrial to present-day changes in tropospheric hydroxyl radical and methane lifetime from the Atmospheric Chemistry and Climate Model Intercomparison Project (ACCMIP). *Atmospheric Chemistry and Physics*, *13*(10), 5277-5298.
Nakagawa, T., Sakurai, T., Nishioka, T., & Touhara, K. (2005). Insect sex-pheromone signals mediated by specific combinations of olfactory receptors. *Science*, *307*(5715), 1638-1642.
Otterstatter, M. C., & Thomson, J. D. (2008). Does pathogen spillover from commercially reared bumble bees threaten wild pollinators? *PLoS One*, *3*(7), e2771.
Papaj, D. R., & Prokopy, R. J. (1989). Ecological and evolutionary aspects of learning in phytophagous insects. *Annual review of entomology*, *34*(1), 315-350.
Pasqualini, S., Tedeschini, E., Frenguelli, G., Wopfner, N., Ferreira, F., D'Amato, G., & Ederli, L. (2011). Ozone affects pollen viability and NAD (P) H oxidase release from Ambrosia artemisiifolia pollen. *Environmental Pollution*, *159*(10), 2823-2830.
Penny, J. H. J. (1983). Nectar guide colour contrast: a possible relationship with pollination strategy. *New phytologist*, *95*(4), 707-721.

Pinto, D. M., Blande, J. D., Nykänen, R., Dong, W. X., Nerg, A. M., & Holopainen, J. K. (2007). Ozone degrades common herbivore-induced plant volatiles: does this affect herbivore prey location by predators and parasitoids?. *Journal of chemical ecology*, *33*(4), 683-694.
Potts, S. G., Biesmeijer, J. C., Kremen, C., Neumann, P., Schweiger, O., & Kunin, W. E. (2010). Global pollinator declines: trends, impacts and drivers. Trends in ecology & evolution, 25(6), 345-353.
Raguso RA (2008) Start making scents: the challenge of integrating chemistry into pollination ecology. Entomol Exp Appl 128:196 207
Rich, S. (1964). Ozone damage to plants. *Annual Review of Phytopathology*, *2*(1), 253-266.
Riffell, J. A., Lei, H., Abrell, L., & Hildebrand, J. G. (2013). Neural basis of a pollinator's buffet: olfactory specialization and learning in Manduca sexta. *Science*, *339*(6116), 200-204
Reinhard, J., Sinclair, M., Srinivasan, M. V., & Claudianos, C. (2010). Honeybees learn odour mixtures via a selection of key odorants. *PloS one*, *5*(2), e9110.
Roddy, A. B., Brodersen, C. R., & Dawson, T. E. (2016). Hydraulic conductance and the maintenance of water balance in flowers. *Plant, cell & environment*, *39*(10), 2123-2132.
Roelofs, W. L., & Comeau, A. (1969). Sex pheromone specificity: taxonomic and evolutionary aspects in Lepidoptera. *Science*, *165*(3891), 398-400.
Roelofs, W.L., A. S. Hill, R. T. Cardé, and T. C. Baker. 1974. Two sex pheromone components of the tobacco budworm moth, Heliothis virescens. Life Sciences 14:1555–1562.
Sánchez-Bayo, F., & Wyckhuys, K. A. (2019). Worldwide decline of the entomofauna: A review of its drivers. Biological Conservation, 232, 8-27.
Sandermann, H., Ernst, D., Heller, W., & Langebartels, C. (1998). Ozone: an abiotic elicitor of plant defence reactions. *Trends in Plant Science*, *3*(2), 47-50.
Sandermann, H. (2008). Ecotoxicology of ozone: bioactivation of extracellular ascorbate. *Biochemical and biophysical research communications*, *366*(2), 271-274.
Schäffler, I., Steiner, K. E., Haid, M., van Berkel, S. S., Gerlach, G., Johnson, S. D., ... & Dötterl, S. (2015). Diacetin, a reliable cue and private communication channel in a specialized pollination system. *Scientific reports*,*5*, 12779.
Schaich, K. M., & Pryor, W. A. (1980). Free radical initiation in proteins and amino acids by ionizing and ultraviolet radiations and lipid oxidation—Part III: Free radical transfer from oxidizing lipids. *Critical Reviews in Food Science & Nutrition*, *13*(3), 189-244.
Schiestl, F. P. (2005). On the success of a swindle: pollination by deception in orchids. *Naturwissenschaften*, *92*(6), 255-264.
Schnell, R. C., Oltmans, S. J., Neely, R. R., Endres, M. S., Molenar, J. V., & White, A. B. (2009). Rapid photochemical production of ozone at high concentrations in a rural site during winter. *Nature Geoscience*, *2*(2), 120-122.
Schoene, K., Franz, J. T., & Masuch, G. (2004). The effect of ozone on pollen development in Lolium perenne L. *Environmental pollution*, *131*(3), 347-354.
Shields, V. D. C., & Hildebrand, J. G. (2001). Responses of a population of antennal olfactory receptor cells in the female moth Manduca sexta to plant-associated volatile organic compounds. *Journal of Comparative Physiology A*, *186*(12), 1135-1151..
Simões, P., Ott, S. R., & Niven, J. E. (2011). Associative olfactory learning in the desert locust, Schistocerca gregaria. *Journal of Experimental Biology*, *214*(15), 2495-2503.
Simonds, V., & Plowright, C. M. S. (2004). How do bumblebees first find flowers? Unlearned approach responses and habituation. *Animal Behaviour*, *67*(3), 379-386.
Slootweg, G., & Van Meeteren, U. (1991, March). Transpiration and stomatal conductance of roses cv sonia grown with supplemental lighting. In Hortifroid, V International

Symposium on Postharvest Physiology of Ornamental Plants; Importance of Cold in Ornamental 298 (pp. 119-126)
Spaethe, J., Tautz, J., & Chittka, L. (2001). Visual constraints in foraging bumblebees: flower size and color affect search time and flight behavior. *Proceedings of the National Academy of Sciences*, *98*(7), 3898-3903
Spivakovsky, C. M., Logan, J. A., Montzka, S. A., Balkanski, Y. J., Foreman-Fowler, M., Jones, D. B. A., & Wofsy, S. C. (2000). Three-dimensional climatological distribution of tropospheric OH: Update and evaluation. *Journal of Geophysical Research: Atmospheres*, *105*(D7), 8931-8980.
Steinbrecht, R. A. (1997). Pore structures in insect olfactory sensilla: a review of data and concepts. *International Journal of Insect Morphology and Embryology*, *26*(3-4), 229-245.
Stevenson, P. C., Nicolson, S. W., & Wright, G. A. (2017). Plant secondary metabolites in nectar: impacts on pollinators and ecological functions. *Functional ecology*, *31*(1), 65-75.
Tan, K. H., & Nishida, R. (2000). Mutual reproductive benefits between a wild orchid, Bulbophyllum patens, and Bactrocera fruit flies via a floral synomone. *Journal of Chemical Ecology*, *26*(2), 533-546
Tepedino, V. J. (1981). The pollination efficiency of the squash bee (Peponapis pruinosa) and the honey bee (Apis mellifera) on summer squash (Cucurbita pepo). *Journal of the Kansas Entomological Society*, 359-377.
Theis, N. (2006). Fragrance of Canada thistle (Cirsium arvense) attracts both floral herbivores and pollinators. *Journal of chemical ecology*, *32*(5), 917-927.
Tollsten, L., & Bergström, L. G. (1993). Fragrance chemotypes of Platanthera (Orchidaceae)-the result of adaptation to pollinating moths?. *Nordic Journal of Botany*, *13*(6), 607-613.
Van Dingenen R, Dentener FJ, Raes F, Krol MC, Emberson L, et al. 2009. The global impact of ozone on agricultural crop yields under current and future air quality legislation. *Atmos. Environ* 43(3): 604–618.
Van Swaay, A. Cuttelod, S. Collins, D.Maes, M.L.p. Munguira, M. ŠaŠić, J. Settele, R. Verovnik, T. Verstrael, M. Warren, M.Wiemers, I. WynhoEuropean Red List of Butterflies Publications Office of the European Union, Luxembourg (2010)
Vickers, N. J., Christensen, T. A., Baker, T. C., & Hildebrand, J. G. (2001). Odour-plume dynamics influence the brain's olfactory code. *Nature*, *410*(6827), 466-470.
Vingarzan, R. (2004). A review of surface ozone background levels and trends. *Atmospheric Environment*, *38*(21), 3431-3442.
Vogel, S. (1962). The role of scent glands in pollination: on the structure and function of osmophores. *New Delhi: Amerind xvi, 202p.-illus.. En Icones. Published for Smithsonian Institute Libraries. Translation by JS Bhatti of Duftdrusen im Dienste der Bestaubung: uber Bau und Funktion der Osmophoren. Mainz: Verlag der Akademie der Wissenschaften und der Literature*
Vogt, R. G., & Riddiford, L. M. (1981). Pheromone binding and inactivation by moth antennae. *Nature*, *293*(5828), 161-163.
Vuorinen T, Nerg A-M, Holopainen JK (2004a) Ozone exposure triggers the emission of herbivore-induced plant volatiles, but does not disturb tritrophic signalling.*Environ Pollut* 131: 305–311
Wallisdevries, M. F., & Van Swaay, C. A. (2006). Global warming and excess nitrogen may induce butterfly decline by microclimatic cooling. *Global Change Biology*, *12*(9), 1620-1626.

Weiner, C. N., Werner, M., Linsenmair, K. E., & Blüthgen, N. (2014). Land-use impacts on plant–pollinator networks: interaction strength and specialization predict pollinator declines. *Ecology*, *95*(2), 466-474.
Winfree, R., Bartomeus, I., & Cariveau, D. P. (2011). Native pollinators in anthropogenic habitats. *Annual Review of Ecology, Evolution, and Systematics*, *42*, 1-22.
Winfree, R., Williams, N. M., Gaines, H., Ascher, J. S., & Kremen, C. (2008). Wild bee pollinators provide the majority of crop visitation across land-use gradients in New Jersey and Pennsylvania, USA. *Journal of applied ecology*, *45*(3), 793-802.
Witton, J. T. (2013). *Plant responses to long term elevated ozone in a British upland semi-natural grassland* (Doctoral dissertation, University of York).
Werker, E. (1993). Function of essential oil-secreting glandular hairs in aromatic plants of Lamiacea—a review. *Flavour and Fragrance Journal*, *8*(5), 249-255.
Wright, G. A., & Thomson, M. G. (2005). Odor perception and the variability in natural odor scenes. *Recent advances in phytochemistry*, *39*, 191-226.
Wright, G. A., Lutmerding, A., Dudareva, N., & Smith, B. H. (2005). Intensity and the ratios of compounds in the scent of snapdragon flowers affect scent discrimination by honeybees (Apis mellifera). *Journal of Comparative Physiology A*, *191*(2), 105-114.
Wright, G. A., & Schiestl, F. P. (2009). The evolution of floral scent: the influence of olfactory learning by insect pollinators on the honest signalling of floral rewards. *Functional Ecology*, *23*(5), 841-851.
Wyatt, T. D. (2014). *Pheromones and animal behavior: chemical signals and signatures*. Cambridge University Press.
Yáñez-Serrano, A. M., Nölscher, A. C., Bourtsoukidis, E., Gomes Alves, E., Ganzeveld, L., Bonn, B., ... & Andreae, M. O. (2018). Monoterpene chemical speciation in a tropical rainforest: variation with season, height, and time of dayat the Amazon Tall Tower Observatory (ATTO). Atmospheric Chemistry and Physics, 18(5), 3403-3418.
Yang, L. H., & Gratton, C. (2014). Insects as drivers of ecosystem processes. *Current Opinion in Insect Science*, *2*, 26-32.*ering Chemistry* 44: 1342.
Yoshida, T. (1975). Lethal effect of ozone gas on the adults of Sitophilus oryzae (Coleoptera: Curculionidae) and Oryzaephilus surinamensis (Colioptera: Cucujidae). *45*(1), 10-15.
Young PJ, et al. (2013), Pre-industrial to end 21st century projections of tropospheric ozone from the Atmospheric Chemistry and Climate Model Intercomparison Project (ACCMIP). *Atmos. Chem. Phys* 13: 2063–2090.

Chapter #1

A hawkmoth copes with pollution-induced oxidation of flower odors by learning the degraded floral plumes

Summary

Pollination strongly contributes to food production and often relies on pollinating insects locating flowers by olfactory cues. However, atmospheric pollution may interfere with pollination by disrupting floral plumes that pollinators use to navigate to flowers. In this study, we examine the impacts of pollution-induced elevated ozone levels on the composition of a floral plume of *Nicotiana alata* and the response of *Manduca sexta* to the ozone-altered plume before and after training. Ozone exposure altered the plume of *N. alata* and disrupted the innate attraction of naïve *M. sexta* to the altered plume. However, associative learning can offset this disruption in attraction. Moths that were enticed with visual cues to an artificial flower emitting an ozonated plume rapidly learned to associate the ozonated plume with a nectar reward. More importantly, moths that were rewarded while experiencing the unozonated plume of their host subsequently found the ozonated plume of the same host attractive, most likely due to experience-based reinforcement of ozone-insensitive cues in the plume.The attraction of moths to both unaltered and ozonated plumes is critical to their toleration of polluted landscapes. At the host plant, where moths feed, floral emissions are relatively pure. As floral plumes travel away from the host, however, they become degraded by pollution. Therefore, following plumes to hosts requires recognizing both conditions of the plume. The ability to generalize between the pure and ozone-altered scents may enable pollinators such as *M. sexta* to maintain communication with their co-evolutionary plant partners and reduce the impact anthropogenic oxidants may have on plant-pollinator systems.

INTRODUCTION

Pollination is integral to maintaining diverse and healthy ecosystems (Kevan 1999), and it strongly contributes to global food production (Klein et al. 2007). The coevolutionary relationship between plants and their pollinators is maintained when plants emit signals that pollinating insects can detect and recognize as belonging to a host plant. These signals include visual cues, such as brightly colored flowers, and olfactory cues-- i.e. floral scents (Kunze and Gumbert 2001). Because the visual acuity of insect pollinators is limited to a resolution of centimeters to a few meters for most flowers (Kapusjansky et al. 2010), smell is likely to be the dominant sensory modality guiding pollinators to flowers over long distances. Floral scents are comprised of bouquets of volatile organic compounds (VOCs) that are emitted from a flower and travel downwind, forming a "scent pathway" that can lead pollinators through the landscape to their host plant.

Animal pollination depends on plants producing signals that are maintained in the landscape and which pollinators can recognize, yet the plume composition of a given plant species may vary both spatially and temporally. Alterations in floral scent over evolutionary time scales, like changes in scent due to modifications in genes coding for specific VOCs, have been shaped by the coevolutionary partnership between plants and pollinators (Dudareva and Pickersky 2000). However, variation in plume production can also occur at sub-evolutionary timescales, and a pollinator will encounter variable plumes over its lifetime, even if it were to forage on just one plant species. Floral plumes can vary due to differences both in emissions and in changes in the plume that occur post-emission as it moves through the atmosphere. A plant's emission of floral plumes can vary over time, due to both diel (e.g. Theis et al. 2007; Raguso et

al. 2003; Muhlemann 2014) and seasonal cycles as plants progress through different phenological states (e.g Theis et al. 2007; Desurmont et al. 2015). Furthermore, production of plumes may vary across space: a population of one species in the landscape may have a different genetic expression for floral scent than another patch of the same species (Knudsen et al. 2006), and environmental gradients such as soil moisture and nutrient load can also influence plume production (Majestic et al. 2009). Post-emission plume transformation also contributes to the variability of floral scents across a landscape: changes to wind speed, temperature, and turbulence all affect the concentration of a plume that the insect experiences and the probability of and frequency at which a pollinator encounters a plume (Murlis et al. 1992; Finelli et al. 2000). Likewise, a pollinator's location in the landscape and its distance from the emitting host plant will dictate the frequency with which it encounters a floral scent (Visser et al. 1986).

Given the variability in floral scents that occurs within the spatial and temporal foraging breadth of a single insect, pollinators must exhibit strategies to cope with plume variation. Pollinators could maintain attraction to variable cues simply by manifesting a broad innate attraction to floral scents, finding many floral compounds innately attractive (e.g Bisch-Knaden et al. 2018), such that a relatively stable subset provides a reliable cue in the midst of variation. If a broad innate recognition of cues is not sufficient to maintain attraction to variable floral scents, pollinators can use another strategy to cope with changing cues: learning. Pollinators can modulate their preference for flowers based on their experience, so that an insect with no innate recognition for a specific floral compound or blend of compounds can associate that olfactory signal with a nectar reward while feeding at the flower (Wright and Schiestl 2009). Learning in this way leads to an increased repertoire of olfactory cues: hawkmoths readily forage on an innately less-preferred host plant after olfactory-association, but they maintain their innate recognition for preferred host flowers (Riffel et al. 2013). Learning may also assist pollinators in

recognizing floral plumes that have been modified as they move downwind of the plant; after learning an odor, honeybees can later recognize that same odor at a lower concentration (Bhagavan and Smith 1997; Pelz et al. 1997). Another type of learning even enables insects to recognize compounds they have no first-hand associative experience with: pollinators may generalize their experience with one cue to another cue they have no experience with, if that compound has a similar chemical structure (Daly et al. 2001). By responding similarly to chemically related compounds, pollinators could follow plumes that differed at production due to plant genetics or physiological conditions or that differed due to modest chemical transformations in the plume as they traveled away from the plant. Thus, both plasticity in behavior and innate recognition of compounds may enable pollinators to cope with variable olfactory cues.

The conditions causing plume variation described above have existed over evolutionary time scales, and thus the composition and concentration of floral plumes produced by plants has been subjected to selection pressures within the overall coevolutionary relationship between plant species and their primary pollinators. Similarly, the ability of pollinators to find rewarding host plants despite variation in chemical signals has been subject to selection pressure. However, pollinators may currently be facing landscapes with steeply increased floral plume variation as a result of anthropogenic interference. Following the industrial revolution, there has been a dramatic increase in the tropospheric load of atmospheric pollutants, including oxidant species nitrate radical, hydroxyl radical, and ozone, which are strongly reactive and can break apart components of a floral plume. All three radicals can react with carbon-carbon double bonds, and the majority of currently examined VOCs are susceptible to oxidation (Atkinson and Arey 2003; Baker et al. 2002). Because tropospheric oxidants can react with many (Lusebrink et al. 2015; McFrederick et al. 2008) but not all (Lusebrink et al. 2015) components in a floral plume,

exposure to tropospheric oxidants could leads to both a decrease in some key compounds and a change in the relative concentrations of individual reactive and non-reactive compounds in a plume (Farre-Armengol et al. 2016; Li et al. 2016)—both of which may be important cues to foraging insects (Bruce et al. 2005). Moreover, when such oxidants react with a VOC, they induce a series of reactions that can lead to the production of secondary compounds. Secondary compounds may themselves be long-lived VOCs but also include compounds which are very dissimilar to the parent VOCs, such as formaldehyde, acetone, and carbon monoxide. (McFrederick et al. 2008; Lee et al. 2006).

Tropospheric oxidants thus have the potential to alter floral scent trails and impede pollinators attempting to locate host plants. Moreover, atmospheric oxidants have increased steeply since the industrial revolution, and may continue to rise in concentration. Ozone has increased from approximately 10ppbv or less in preindustrial times (Hauglustaine and Brasseur 2001) to current averages in North America of 20-45ppbv (Vingarzan 2004), with spikes as high as 120 ppbv during summertime ozone events (Fiore 2002; Vingarzan 2004). Hydroxyl radical, which contributes the most of any chemical to atmospheric oxidation, has increased from a production rate of approximately 116Tmol/year in preindustrial times to ~188Tmol/year produced currently (Monks 2005). To continue using floral scents as cues in a world with elevated tropospheric oxidants, pollinators must either cue in on non-reactive volatile compounds, or they must learn the succession of odors they encounter in the landscape, ranging from highly altered plumes at distances far from the host plant, to plumes that are unaltered at the flower. Current work has established that floral plumes altered by the oxidant ozone are less attractive than unaltered plumes to a variety of insects including a bumblebee and two specialist herbivores (Farre-armengol 2016; Fuentes et al. 2013; Li et al. 2016). Chemical modelling studies have predicted that ozone and hydroxyl radical will react with floral plumes across

landscapes (McFrederick et al. 2007, 2008 & 2009) and that as a result, insects will be less adept at locating their host plants in oxidant-enriched environments (Fuentes et al. 2016). While these works have demonstrated the potential for tropospheric oxidants to alter floral plumes and impede insect foraging, neither empirical nor computational studies have considered the ability of insects to learn to identify and respond to oxidant-altered plumes, nor have any tested the response of an insect to hydroxly-altered plumes. Can the flexibility in cue recognition or the learning abilities of pollinators, established to help these insects thrive in variable landscapes, assist them in recognizing floral cues even as air pollution alters the integrity of those plumes?

We tested the ability of one nighttime pollinator, the hawkmoth *Manudca sexta*, to recognize and learn ozone-altered and hydroxyl-altered plumes of one of its preferred host flowers, *Nicotiana alata*, using these oxidants as approximation for the nighttime dominant nitrate radical. After demonstrating that ozone and hydroxyl radical substantially alters the odor profile of *N. alata* and renders it unattractive to naive moths, we develop an odor learning protocol for *M. sexta,* testing the abilities of moths to associate oxidant-altered plumes with rewards. We further consider two possible learning scenarios using only the oxidant ozone in which *M. sexta* could navigate to its host using odor cues, despite the alteration of the floral plume by ozone. First, we test whether *M. sexta* can can learn to use an ozonated plume as a cue that leads to a pure plume at a rewarding artificial flower. Second, we test whether experience at an unpolluted host broadens the suite of cues used in host recognition such that the plume remains attractive despite ozonation.

METHODS

Study Organisms Experiment Preparation

Manduca sexta were raised in a temperature and light controlled chamber (light:dark= 16:8, 70% relative humidity and 25° C during the light phase, and 60% relative humidity and 20 °C during the dark phase) so that the moths experienced nighttime conditions during the day, and were active during normal working hours of the researchers. *Manduca sexta* larvae were reared on artificial diet, and wandering larvae were placed in cylinders smoothly drilled in wooden blocks to pupate.

N. alata flowers used for odor collection and for behavior tests in the wind tunnel were reared in temperature and light-controlled chamber with the same light and temperature specifications used for the *M. sexta* rearing chamber (light:dark= 16:8, and 25° C during the light phase, humidity and 20 °C during the dark phase).

Effect of increased ozone levels on flower plume chemistry

We examined the effects of ozone on the plume composition of *Nicotiana alata* by comparing the plume mixed with scrubbed air to the plume mixed with air enriched with 120 ppbv ozone. Here, ozone serves as a conservative estimate for the nighttime dominant nitrate radical, as both ozone and nitrate can break apart carbon-carbon double bonds, but ozone is typically less reactive than nitrate radical. We placed two one-day old *N. alata* flowers into a small chamber (volume, 200 ml). Air that had been run through a charcoal scrubber was blown into the chamber containing the two flowers at a rate of approximately 3l/min. The floral headspace was then pulled from the chamber via two pumps, each pulling a little over 1l/min. Both of the split floral headspaces were run through a flow meter and then into an airtight 2l glass bottle at a rate of ~1l/min. In one glass bottle, scrubbed air at a rate .5l/min was also mixed into the bottle. In the other glass bottle, .5l/min of ozonized air at a concentration just above 120 ppbv was mixed with the floral headspace: this concentration was set so that the ozone concentration in the mixed

plume entering the wind tunnel was ~120 ppbv. All ozone used in these experiments was generated using a Thermo Scientific Ozone generator Model 165 (Thermo Scientific in., Pittsburg PA) and ozone concentrations were measured using an ozone analyzer (Model 202, 2B Technologies INc., Boulder CO). Both the plume mixed with scrubbed air and the plume mixed with ozone-enriched air (120 ppbv) were mixed through a further series of two 1l bottles, before passing through a small box containing five 5mm PDMS tubes that captured the floral plumes as it passed over them. This collection allowed direct comparison between the plume mixed with air and mixed with ozone from the same flowers over the same mixing time.

Following the scent collection, PDMS tubes were analyzed individually using a thermo desorption unit (TDU, Gerstel, Germany) coupled to a temperature-programmable vaporizing unit (CIS 4, Gerstel, Germany), which was linked to an Agilent 7890A gas chromatograph (Agilent Technologies, CA) running in splitless mode and being connected to an Agilent 5975C mass spectrometer (electron impact mode, 70eV, ion source: 230◦C, quadrupole: 150°C,mass scan range: 33–350u). We used a nonpolar column (HP-5 MS UI, 30m length, 0.25mm ID, 0.25µm film thickness, J and W Scientific, USA) under constant helium flow of 1.1ml/min. The TDU temperature raised from 30°C to 200°C at a rate of 100°C/min and held for 5min. Volatized compounds were trapped within the CIS 4 cooled injection system at -50°C and subsequently injected into the GC. The GC oven was programmed to hold 40°C for 3min, to increase the temperature at 5°C/min to 200°C, then to increase temperature at 20°C/min to 260°C, which was kept for 15min. Unprocessed data files were then exported and analyzed using the software package XCMS (Smith et al., 2006) implemented in R (R Core Team, 2014). Peak area values were log transformed to ensure normality and finally compared by a principal component analysis

Effect of increased hydroxyl levels on flower plume chemistry

We further assessed the impacts of hydroxyl radical on the plume of *N. alata.* Hydroxyl radical was tested because it is more reactive than ozone, with Nitrate radical, the pollutant prevalent during the nighttime, having a reactivity between ozone and hydroxyl radicals' intermediary reactivity for many VOC compounds (Atkinson and Arey 2003). Thus, by testing the impacts of both ozone and hydroxyl radical on a floral plume, we sought to establish a window of potential reactiveness of nitrate radical. As with ozone, we reacted plumes of *Nicotiana alata* with hydroxyl radical. Hydroxyl radical was generated by flowing humid air past a UVB lamp in a specialized glass mixing chamber: the flow of the newly-formed hydroxyl radical met and reacted with the plume of *Nicotiana alata.* The hydroxyl radical reaction had been tested at Max Planck Institute for Chemistry, assessing the real-time loss of a known concentration of limonene as it reacted with the hydroxyl radical, using a PTRMS. Generation of hydroxyl radical could be loosely controlled by increasing or decreasing the rate of airflow air and concentration of humidity in the humidified air that flowed over the UVB lamp, and partially covering the lamp with electrical tape: however, this was not precise, and reaction with limonene was primarily meant to assess that hydroxyl radical was indeed being generated. The specialized glass mixing chamber for generating hydroxly radical, but not the PTRMS, was then transferred to Max Planck Institute for Chemical Ecology for behavioral tests with *Manduca sexta.*

Both pure and hydroxyl-exposed plumes were generated in a manner similar to the generation of pure and ozone-exposed plumes. 3l/min of scrubbed clean air was blown through a small chamber (volume 200ml) containing two one-day old *N. alata* flowers. The floral headspace was then pulled from the chamber via two pumps, each pulling a little over 1l/min. Half of the floral headspace was directed through a flow meter and into an airtight 2l glass bottle

at a rate of ~1l/min, where .25l/min of scrubbed air was added, creating the pure plume headspace.

The other half of the floral headspace was passed through a flow-meter and blown directly into the glass reaction chamber so that the plume was added less than three centimeters from the UVB lamp which generated the hydroxyl radical. The flow of humidified air passing over the UVB lamp was .25l/min. The flow from the glass mixing bottle, containing both the humidified air with the hydroxyl radical and the floral plume, was then directed into a 2l bottle so that the mixing time and exposure to the volume of glass was the same as the plume without hydroxyl radical. As with pure and ozone-exposed floral plumes, both the plume mixed with scrubbed air and the plume mixed with hydroxyl radical enriched air (of an unknown concentration) were mixed through a further series of two 1l bottles, before passing through a small box containing five 5mm PDMS tubes that captured the air and hydroxyl-exposed plumes. PDMS tubes were then analyzed for chemical composition of floral plumes using the same protocol described in '*Effect of increased ozone levels on flower plume chemistry*' for floral plumes exposed to air or ozone-enriched air.

Behavioral Assays

For all behavior assays, three-day old naïve *M. sexta* were collected from their climate chamber in individual mesh containers and given at least one hour to acclimate to the conditions in the wind tunnel in an adjacent room. The wind tunnel itself is a plexiglas structure with dimensions L x H x W: 250 x 90 x 90 cm with dark circles randomly scattered on the tunnel floor that give visual feedback for flight stabilization. To start each behavior assay, a moth was placed inside the wind tunnel perched on the lid of its mesh container on a stand at the downwind side of the wind tunnel. Moths were mildly provoked to initiate flight by shaking the lid of the mesh

container on which they were standing. Once flight was initiated, the moths were given four minutes to complete their behavior assay, during which time they were filmed by a series of cameras inside and outside of the wind tunnel. Scents were brought into the wind tunnel via Teflon tubing that was passed into upright metal poles. In some tests, artificial flowers were added atop these upright metal poles, and in others the visual cues were minimized and no artificial flowers were presented. The amount of time that the moths spent investigating a scent source with their extended proboscis was recorded manually.

Manduca sexta*'s innate response to unaltered vs. ozone-altered or hydroxyl-altered plumes of* N. alata

Initially, *M. sexta* were presented with the choice of two artificial flowers made of light blue paper atop metal poles which contained Teflon tubes through which the flow of the various plumes being tested were transported into the wind tunnel. The artificial flowers were located side-by-side at the up-wind side of the wind tunnel. We first tested to see if the moths were innately deterred by either ozone or hydroxyl-radical enriched air. To test this, one of the two artificial flowers spaced ~38cm apart exuded air enriched with either 120 ppbv ozone or enriched with an unknown concentration of hydroxyl radical, and the other emitted air. Air for both sources had been passed through a charcoal scrubber before treatment, and both flowed from the artificial flowers at rate of 0.5l/min. Moths were given four minutes of flight in the wind tunnel to investigate the two paper 'flowers', and the amount of time moths spent at each 'flower' was recorded.

Next, we tested the moths innate attraction to the oxidant altered plumes compared to the natural plume of *N. alata*. First, we tested the impact of ozone on the plume attractiveness: the plume of two *N. alata* flowers was split and half was ozonized by mixing with ozone thorough a

series of bottles or mixed only with scrubbed air, following the protocol described in 'the effects of ozone on plume composition.' The ozonized and pure plume were entered into the wind tunnel a rate of 0.5l/min. Again, moths were given four minutes of flight in the wind tunnel to investigate the two paper 'flowers', and the amount of time moths spent investigating each 'flower' with an extended proboscis was recorded. This same behavior test was later repeated with hydroxyl-exposed plumes instead of ozone-exposed plumes.

Learning protocol for Manduca sexta

Learning may play an important role in helping pollinators locate their host plants, and may assist them in environments with elevated tropospheric oxidants, if they can learn oxidant-altered plumes.To test the learning ability of *M. sexta*, we first established a simple three-step olfactory learning procedure for *M. sexta* using the single odorant linalool (Fig.2A).In step one, we assessed *M. sexta*'s innate response to the single odorant linalool. Moths were placed at the base of the wind tunnel and presented with two options: 12ul of 10^{-2} linalool in mineral oil, flowing at a rate of 0.5l/min from a 250ml airtight bottle, or .5l/min of scrubbed air. The linalool plume and scrubbed air entered the wind tunnel via the Teflon tubes inside upright metal poles: the structure was the same as used in innate tests, except that there was no artificial flower atop the metal pole. In this initial test without strong visual cues, moths typically did not investigate either plume, which is in agreement with Bisch-Knaden et al. (2018) which finds that linalool is not innately attractive to foraging *M. sexta*. After a fifteen-minute rest period, the same moth was returned to the wind tunnel for training: the wind tunnel now contained a light blue paper 'flower,' centered in the back of the wind tunnel emitting the same 0.5l/min of the linalool odor, and with 12ul of 30% sucrose solution in an Eppendorf tube at the center of the flower. Moths were given four minutes to forage on the artificial flower. Those moths that successfully

foraged—feeding or attempting to feed after all the sucrose had been consumed for one minute or more—were removed from the wind tunnel and given another 15 minute rest interval before being returned to the wind tunnel to repeat their initial air. vs. linalool choice test, again without obvious visual cues.

To ensure that moths had learned linalool and were not merely more responsive to any scent presented after successfully foraging, we switched the training compound—the scent presented with the rewarding artificial flower—to 2-phenyl-ethanol. 2-phenyl-ethanol was selected as it has been found to be attractive to female *M. sexta* (Bisch-Knadden et al 2018) and thus were easily enticed to visit the artificial flower with the rewarding sucrose reward. In this test, moths were first flown in the wind tunnel towards a plume of the linalool odor and a plume of air, without visual cues as before. After this initial flight, moths were rested and then returned to the wind tunnel where 2-phenyl-ethanol was emitted from an artificial flower containing a 30% sucrose solution. Those moths that fed on the rewarding artificial flower emitting the 2-phenyl-ethanol scent were returned to the wind tunnel again to re-assess their response to linalool vs. air without obvious visual cues. This three-step procedure was used in all further learning assays, with the training scent changes to assess different types of learning.

Direct learning of ozone-altered and hydroxyl-altered floral scents

With a learning system thus established for a single odorant, we proceeded to test *M. sexta's* ability to directly learn hydroxyl-reacted and ozonized plumes. Following the same three-step learning procedure used for linalool, we tested *M. sexta'*s initial response to the ozone-altered or hydroxyl-radical exposed plume vs. air, and then reassessed that response after the moths had foraged on an artificial flower emitting the ozone-altered or the hydroxyl-radical exposed plume *N. alata* plume, respectively.

Learning ozonized plumes via a more realistic mechanism

Close to the flower, floral volatiles have been exposed to oxidants for only a short amount of time, resulting in a negligible level of alteration of the plume. While moving away from the source through a polluted atmosphere however, the plume will become more and more altered. Can moths learn that oxidized plumes lead to rewarding flowers, even though they experience a natural plume at the moment that they receive a nectar reward? To answer this question, we tested two different learning protocols, changing the scent presented with the rewarding artificial flower in the training step. These further tests probing mechanisms of learning were conducted only with ozone-altered (not hydroxyl-radical-reacted) plumes.

First, we tested to see if *M. sexta* could learn ozone-altered plumes if the ozone-altered plume preceded the unaltered plume at the rewarding artificial flower in the training step. Using the same initial and final learning tests used for direct learning tests, we modified our training phase so that the moth was given one minute to fly towards the artificial flower emitting the ozonized plume of *N. alata* at an increased rate of 1l/min, until the moth's extended proboscis was ~8cm away from the source. At this point, we instantaneously switched the scent of the artificial flower from the ozonized plume to the to the unaltered plume using a manually-activated solenoid valve. Moths could then feed at the artificial flower only when it was emitting the unaltered plume. Both the decrease in time allotted for investigation to one minute and the increase in flow of the floral scent from the artificial flower were used to entice the moth to hone in on the floral plume so that the approach was obvious and could be well anticipated by the researcher manually shifting the scent flow at the flower from the ozonized to non-ozonized scent (Fig.3B).

Because the ozone-altered and unaltered plume have some overlapping compounds, it is not clear if *M. sexta* can learn a compound if it is preceded by an unrelated compound, or if it relies on the similarities between the preceding and rewarding plume. To remove this potentially confounding variable, we tested *M. sexta'*s ability to learn a single odor presented in the same two-scent training paradigm, in which linalool (a scent not innately attractive to *M. sexta* (Riffel et al. 2009; Bisch-Knaden 2018) led to a rewarding artificial flower emitting 2-phenylethanol (an innately attractive scent Bisch-Knaden 2018). The concentration of both linalool and 2-phenyl-ethanol was 10ul of 10^{-2} odor in mineral oil from 250ml volume airtight glass bottles.

M. sexta foraging in the two-scent learning protocol may not depend on the ozone-altered plume preceding the pure plume. To test whether the ozone-altered plume was redundant or otherwise unnecessary to learning, we next ran a final behavior assay where we removed the ozonized plume from the training step entirely. We tested the moths ability to generalize from the unaltered plume to the ozonized plume after foraging at the unaltered plume. Following the same three-step learning procedure described above, moths were tested for their innate response to the ozonized plume vs. air, but were then trained by feeding at an artificial flower emitting only the original plume (i.e. they did not experience the ozonized plume in the training phase). Individual moth's response to the ozonized plume was then reassessed after this training.

RESULTS

Effects of ozone on the floral headspace of N. alata

Exposing the headspace of *N. alata* flowers to 120 ppbv ozone substantially changed the odor profile of the floral plume (Fig.1A). The primary difference was a reduction in several compounds in the unaltered plume as well as an increase in decenal. These differences yielded

significant separation in the overall plume composition as shown by principal component analysis (Fig.1B).

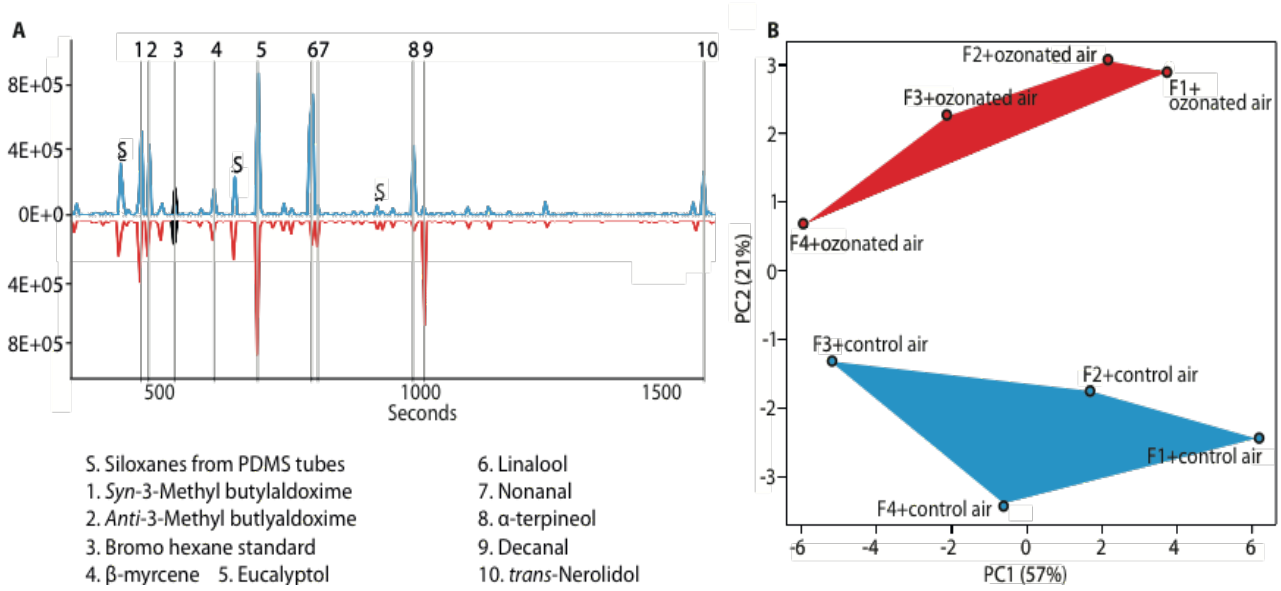

Fig. 1. Plumes of *N. alata* are altered by exposure to ozone. A. Example traces of original (blue) and ozonated (red) split headspaces of two *N. alata* flowers. Numbered peaks are identified by the NIST library (R-match > 90%). B. Principal component analysis of original and ozonated headspaces of four *N. alata* flowers (F1-F4).

M. sexta *innate response to ozone-altered plume and ozone*

After determining that the plume of *N. alata* was altered by 120 ppbv ozone, we tested the innate preference for *M. sexta* to plumes of *N. alata* mixed with air vs. mixed with ozone-enriched air (Fig.2A). Moths preferred the original plume of *N. alata* to the ozonated one: they spent significantly more time probing the artificial flower emitting the original plume relative to the artificial flower emitting the ozonated plume (Fig.2B). The decrease in attraction to the ozone-

altered plume relative to the original plume was not driven by aversion to ozone, as moths in the wind tunnel investigated artificial flowers emitting ozone at 120 ppbv versus scrubbed clean air at the same rate (Fig.2C).

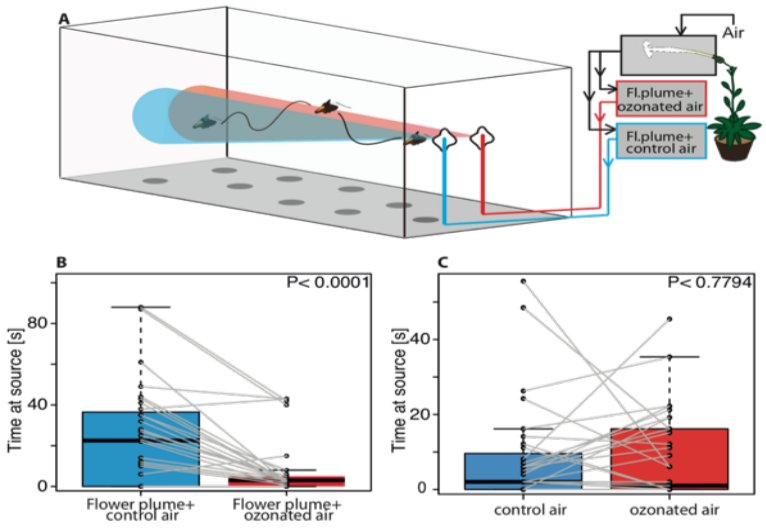

Fig. 2. Moths innately prefer original to ozonated plumes of *N. alata.* A. Schematic of the test for innate preference. B. Time moths spent probing with their proboscis at the artificial flower emitting the original and the ozonized plumes. (Wilcoxon signed rank test, N=31, p<.05). C. Time moths spent probing with their proboscis at the flower emitting scrubbed air and ozonated air (Wilcoxon signed rank test, N=36, p<.05) (Boxplots, 25% and 75% quartiles and median; whiskers, maximum and minimum values; black circles with connecting lines, paired data of individual moths).

Behavioral learning paradigms for M. sexta *foraging on ozone-altered plumes*

Once a learning protocol was developed using a single odor (Fig.S1), we tested whether moths could learn the ozone-altered plume using three different learning paradigms. In all three learning paradigms, the initial and final tests – response to the ozone-altered plume vs. air without visual cues – remained the same, while the training step was altered (Fig.3A-C). Moths

learned the ozone-altered plume when the ozone altered plume was presented at a rewarding artificial flower, i.e., spent significantly more time at the ozonated plume after the training (direct training, Fig 3A1). Moths were also able to learn the ozone-altered plume when the ozone-altered plume preceded the unaltered plume at a rewarding artificial flower in a sequence (Fig.3B1). When tested on their ability to learn a sequence of two single odors rather than plumes, however, the moths only slightly increased their response to Linalool when their subsequent reward was paired with 2-Phenylethanol (Fig.3B1). Finally, even when the moths only experienced the original plume (the ozonated plume being completely absent during the training), they still increased the time they spent investigating the ozonated plume in the subsequent test situation, indicating an ability to generalize learned odors beyond their innately attractive components (Fig.3C1).

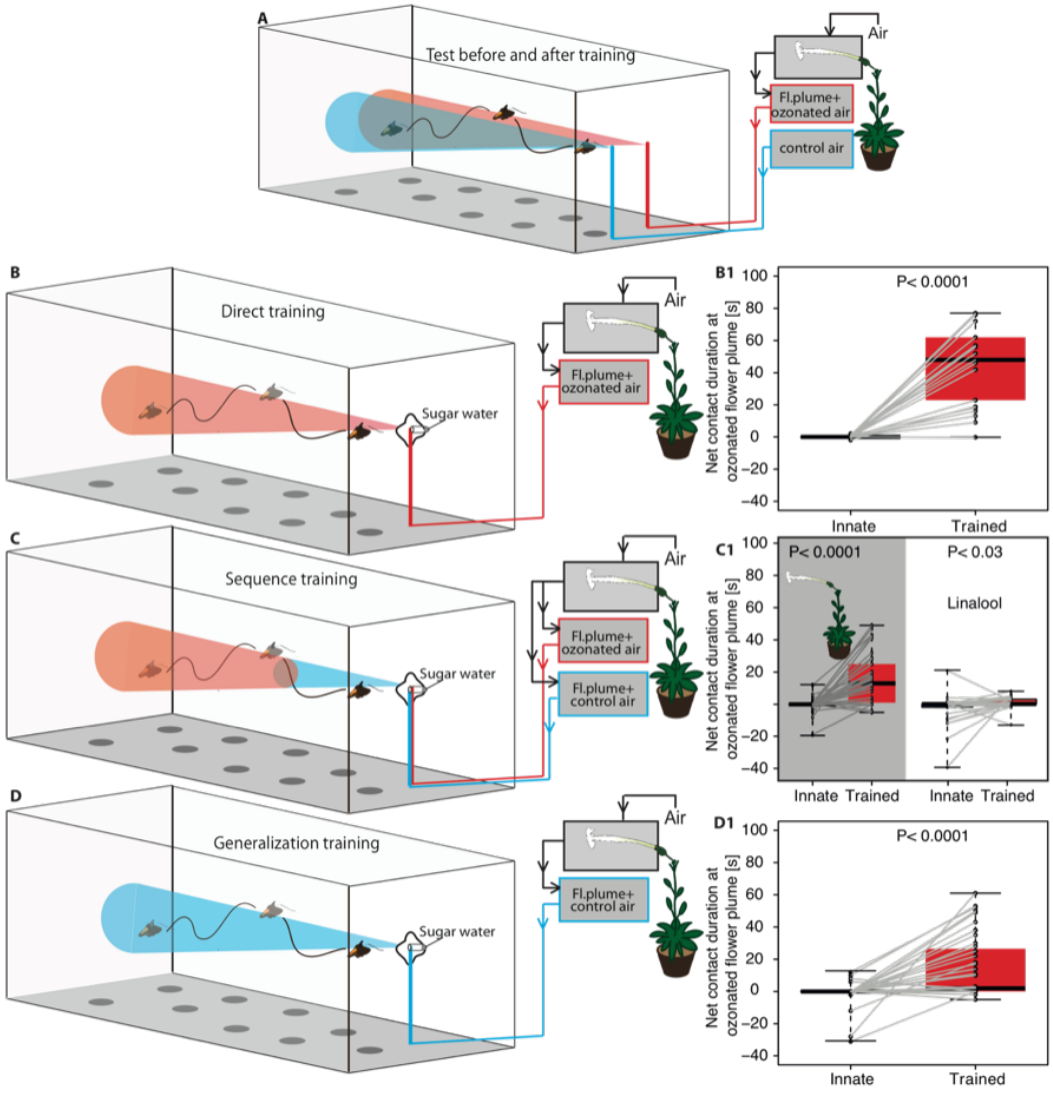

Fig. 3. Moths can learn ozonated plumes. A. Test situation before and after training sessions. B. Direct training, where moths are rewarded with sugar water at an artificial flower emitting an ozonated flower plume. B1. Net contact duration at ozonated flower plume before and after training (Net contact duration; time at ozonated plume minus time at clean air source [s]) (Wilcoxon signed rank test, N=22). C. Sequence training, where moths first follow an ozonated flower plume (red), but then but become rewarded with sugar water in the presence of the original plume (blue). C1. Left boxplots, net contact duration at ozonated flower plume before and after training (N=45), right boxplots, net contact duration at Linalool, when moth in the training situation first had to follow a Linalool plume, but were rewarded at a flower emitting 2-

Phenyl-ethanol) (N=30). D. Generalization training, where moths experience only the original flower plume during the full training situation. D1. Net contact duration at ozonated flower plume before and after training (N=48). (Boxplots, 25% and 75% quartiles and median; whiskers, maximum and minimum values; black circles with connecting lines, paired data of individual moths).

Effects of hydroxyl radical on N. alata *plume and* M. sexta *learning OH-altered plumes*

Plumes of *N. alata* were altered by exposure to hydroxyl radical, although variation in hydroxyl-reacted plumes was very strong, resulting likely from the uncertainty in exact concentration of hydroxyl radical produced. *M. sexta r*esponding to hydroxyl-reacted floral plumes of *N.alata* did not initially find the plumes attractive, but were able to recognize the hydroxyl-altered plumes after direct olfactory training.

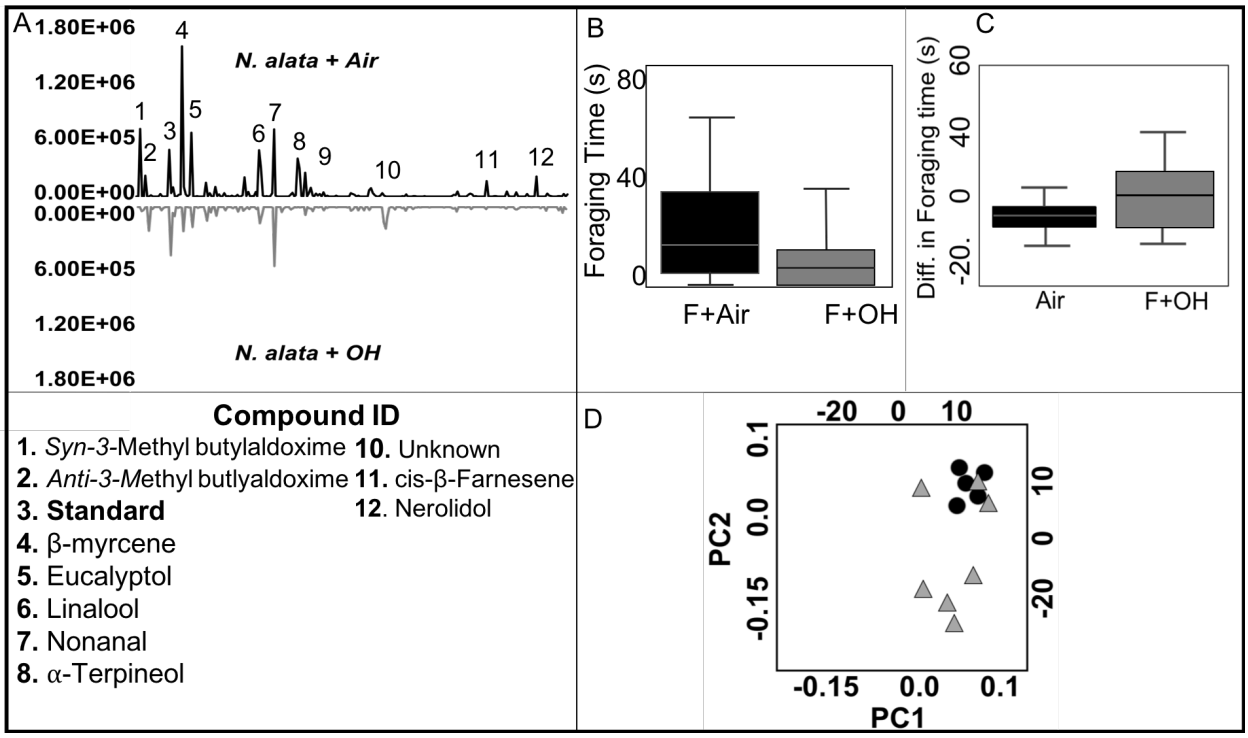

Fig. 4. Plumes of *N. alata* are altered by exposure to hydroxyl radical. **A.** Example traces of original (black) and exposed to OH radical (grey) split headspaces of two *N. alata* flowers. Numbered peaks are identified by the NIST library (R-match > 90%) **B.** Foraging time for *M. sexta* on artificial flowers emitting either *N. alata* plume mixed with air, or *N. alata* plume mixed with hydroxyl radical.**C.** Difference in foraging time on air and plumes mixed with hydroxyl radical before and after training **D.** Principal component analysis of original and hydroxyl-radical exposed headspaces of seven *N. alata* flowers.

DISCUSSION

Ozone alters plumes and diminishes plume attraction to naïve M. sexta

Ozone at concentrations currently reached in the Northern Hemisphere can substantially alter a plume of *Nicotiana alata* (Fig.1A), a result congruent with earlier modeling and empirical studies (McFrederick et al. 2009; Farre-Armengol et al. 2016). The *N. alata* plume mixed only with scrubbed air closely resembles the scent profile previously reported for *N. alata* (Raguso et al. 2003), while the ozone-exposed plume showed a decrease in some alkenes, including the monoterpene **β**-Myrcene and the oxygenate monoterpene Linalool, which were reduced via reaction with ozone (Atkinson and Arey 2003). Other compounds in the plume were not affected by ozonation, including Eucalyptol, which has previously been described as non-reactive with ozone (Destaillats et al. 2005). Because some compounds react with ozone but not others, the relative ratios of compounds in the plume, and thus the overall plume odor for organisms sensitive to those particular compounds, was altered by ozonation (Fig.1B). No new secondary compounds resulting from ozonation of the plume were identified, but a solvent delay in the GCMS protocol prevented low molecular weight compounds such as Acetone, Formaldehyde, or

carbon monoxide from being detected. Had these common secondary compounds been detected, the difference between the ozonated and non-ozonated plume would likely have been even greater.

The *N. alata* plume mixed with hydroxyl radical was severely reduced, showing a marked reduction in concentration and for the majority of floral volatiles, with some floral volatiles even disappearing from the plume after exposure to hydroxyl radical (Fig.4A). Our findings that the plume was more severely altered by exposure to hydroxyl-radical than by exposure to ozone could be explained by hydroxyl radical being typically more reactive than ozone with, as it can break O-H and C-H bonds (Atkinson et al. 1984) in addition to breaking apart C-C double bonds. Another likely explanation is that while ozone concentrations were controlled to reflect realistic tropospheric ozone concentrations, the concentration of hydroxyl radical was not controlled.However, by trial and error adjusting the humidity and flow of the air used for hydroxyl radical generation, we were able to establish a hydroxyl radical concentration that was sufficient to react with some floral volatiles, but not so high as to cause a complete absence of floral volatiles in the plume.

Both ozone and hydroxyl-radical induced alterations of the plume was severe enough to reduce *M. sexta's* innate attraction to the plume of *N. alata* (Fig.2B; Fig.4C).This may be due to any of the following changes to the plume: a decrease or absence of reactive, innately attractive compounds, a resultant alteration in the ratios of compounds – ratios of odors may be critical for insect recognition of scents (Bruce et al. 2005) – or the addition of secondary compounds that may be repellent (Li et al. 2016). Additionally, the presence of oxidants themselves may contribute to this diminished attraction: even though ozone and hydroxyl radical by themselves were not a deterrent to *M. sexta* in our test trial (Fig.2C), a previous study found that *Apis mellifera* were less responsive to compounds presented in ozone vs. air (Dotterl et al. 2016).

Regardless of the mechanism behind the loss of attraction, *M. sexta's* preference for the original plume contributes another case to the body of literature showing that tropospheric oxidants can disrupt innate behavioral responses to attractive compounds (Farre-Armengol et al. 2016; Fuentes et al. 2013; Li et al. 2016).

Manduca sexta *can learn to be attracted to ozonated and hydroxyl-altered plumes*

Although *M. sexta's* innate attraction to the plume of *N. alata* is disrupted by ozone and hydroxyl radical, associative learning enabled *M. sexta* to find the oxidant-altered *N.alata* plumes attractive. *Manduca sexta* readily learned both the ozone-exposed and hydroxyl-exposed plumes after it had foraged on a sucrose reward while being exposed simultaneously to the oxidant-altered plume (Fig.3B).While our results using the oxidant hydroxyl radical can only be considered preliminary given the lack of control of the hydroxyl radical concentration, *M. sexta's* ability to learn even hydroxyl-altered plumes, many of which were strongly reduced in both volume and abundance of different floral volatiles, demonstrates the moth's proficiency in learning olfactory cues. The mechanism of directly associating rewards with ozone or hydroxyl - radical altered plumes is in agreement with existing literature demonstrating that *M. sexta* can directly learn individual compounds (Daly 2000) and complex floral plumes (Riffel et al. 2014) with a reward, and demonstrate subsequent attraction to those odors. However, this direct-associative learning mechanism, while providing evidence that oxidant-altered plumes can be learned by *M. sexta*, would not be sufficient for *M. sexta* to recognize plumes altered by air pollution in the field. In the field, a floral plume becomes gradually altered by tropospheric oxidants as it moves away from the flower, so that a foraging pollinator would never have the opportunity to forage at a flower while being exposed to the altered plume.

In order to learn plumes altered by oxidants in the field, *M. sexta* would have to learn the oxidant-altered plume decoupled from a reward. Such learning could be accomplished via two mechanisms: a moth could experience the oxidant-altered plume at some distance from the flower, followed by an original plume at the flower, and link the olfactory information from the two plumes – i.e. the moths would learn a sequence of oxidant-altered and original plumes. Learning a sequence of two odors leading to a reward has been achieved for honeybees (Hussaini et al. 2007). Alternatively, feeding at the pure plume could reinforce those compounds in the plume that do not become affected by oxidants (e.g. Eucalyptol which is unreactive with ozone in Fig. 1A), and a moth could later recognize these unreactive compounds in the ozonated plume, even if these compounds were not innately attractive – i.e., the moths would generalize from original to oxidant-altered plumes. *Manduca sexta* that experienced an ozonated plume followed immediately by the original plume at a rewarding paper flower, learned to use the ozonated plume as a foraging cue (Fig.3C1 left), which is consistent with learning a sequence of two plumes. When, however, we tested this ability to link two odors more explicitly by using the individual compounds Linalool and 2-Phenyl-ethanol as sequential odor cues, *M. sexta* was unable to learn this sequence (Fig.3C1 right). Thus, the learning of a sequence of odors does not seem to account for *M. sexta*'s ability to learn the ozonated plume in the sequence training paradigm.

Instead of learning a sequence of two scents, moths may have learned by generalizing olfactory information from the original plume to the ozonated plume. Indeed, *Manduca sexta* did not need to experience the ozonated plume in the training session at all to later become attracted to the ozonated plume: merely feeding at an artificial flower emitting the original plume resulted in a subsequent increased attraction to the ozonated plume (Fig.3D1). It is apparent that *M. sexta* has learned to recognize identical or structurally similar compounds between the ozonated and

original plume, rather than linking the sequence of these plumes when approaching the flower. These reinforced compounds could either be those parts of the plume that are not-reactive with ozone (including common flower odors such as Benzaldehyde and Eucalyptol, Raguso et al. 2003), or could be compounds in the ozonated plume that are chemically similar (Daly et al. 2001) to compounds in the original plume that the moth experienced. Directly learning some parts of the plume that are non-reactive is possible, as insects can learn some plume components after associating a full plume with rewards (Reinhard et al. 2010), but learning would then depend on the probability that compounds learned are uncreative with oxidants, assuming that insect learning of odors occurs irrespective of those odors reactivity with oxidants. Alternatively, *Manduca sexta* may generalize from compounds reinforced at the plume to compounds with similar chemical structure in the ozone-altered plume: after learning an initial compound, *M. sexta* can recognize compounds with similar chemical structure that have not been learned, with a decreasing responsiveness to compounds that are progressively less similar (Daly et al. 2001*)*. Chemically similar compounds may activate the same receptors on an insect antenna (Shields and Hildebrand 2001)--e.g. geraniol and linalool (Raguso and Willis 1995) are similarly structured and activate some of the same receptors in *M. sexta,* and a moth may associate a reward with the activation of that olfactory receptor, regardless of what compound was responsible for its activation. Olfactory receptors for *M. sexta* include broadly tuned receptors that can accept odorants from different chemical classes, in addition to highly specific olfactory receptors that binding with just a few key odorants such as sex pheromones (Shields and Hildebrand 2001;Kaissling et al 1989) Olfactory receptors on insect antenna can have different levels of specificity for odorants, *Manduca sexta* may thus recognize ozonated plumes after foraging on original plumes without needing to directly associate non-reactive compounds with

rewards. Regardless of which mechanism is being used, this outcome represents a potentially robust flexibility in foraging on preferred host plants in a polluted environment.

Although learning enables *M. sexta* to recognize an ozonated plume in a manner that it may employ in the field, learning as a means of mitigating effects of anthropogenically-induced plume perturbation is not without limits. To begin, ozone and hydroxyl radical are is just two air pollutants that have increased as a result of anthropogenically driven fossil fuel combustion: nitrate radical, which peaks during the day, has also risen due to increased nitrogen oxides in the atmosphere. In this study, ozone and hydroxyl radical were used as an approximation for nitrate radical, but while both ozone and hydroxyl radical can oxidize alkenes as does nitrate radical, the specific compound chemistry is individual (Atkinson and Arey 2003). Furthermore, to associatively learn a plume, an insect must first locate a host plant; if floral plumes are degraded at long distances this can only be accomplished when an insect comes by chance within either close range or sight of a flower, or if it relies on floral compounds that are not reactive with tropospheric oxidants. An insect's ability to both detect and learn olfactory cues, however, is highly species-specific and restricted by the insect's specific neuro-physiological makeup that is determined by its co-evolutionary history with given host plants. Specialist insects feed on just a few taxonomically related plant species, and naïve specialists may rely on unique and taxonomically-distinctive floral volatiles to ensure they can find their host plants (Brandt et al. 2017; Schaffler et al. 2015; Burger et al. 2010; Milet-Pinheiro et al. 2012). Thus, a specialist insect's foraging success in polluted environments may depend on the oxodiant-reactivity of the few floral compounds that it innately relies on, the similarity of oxidant-induced breakdown compounds to these parent floral volatiles, or on specialist's currently largely untested ability to learn olfactory cues.

In summary, learning can provide one means by which pollinators can still use oxidized floral plumes, and highlights the importance of learning in plant-pollinator interactions. However, the importance of learning as a means of coping with polluted plumes in the field remains to be tested. Learning capabilities are likely to be highly variable among species, and, hence, elevated tropospheric oxidants still pose a potentially serious threat to foraging pollinators. Multiple studies have reported declines in insect abundance and pollinator health in regions across the globe (Potts et al. 2010; Kluser et al. 2007; Fox et al. 2013; Hallman et al. 2018), and various anthropogenic drivers have been implicated in this decline. Anthropogenically elevated air pollution could be another stressor contributing to overall global insect declines. Future work is needed to assess the real threat of oxidants on foraging insects, and such work must consider pollinators as agents capable of plastic behavioral responses in the field.

Citations

Atkinson, R., & Arey, J. (2003). Gas-phase tropospheric chemistry of biogenic volatile organic compounds: a review. *Atmospheric Environment*, *37*, 197-219.

Baker, J., Aschmann, S. M., Arey, J., & Atkinson, R. (2002). Reactions of stabilized criegee intermediates from the gas-phase reactions of O3 with selected alkenes. *International journal of chemical kinetics*, *34*(2), 73-85.

Bisch-Knaden, S., Dahake, A., Sachse, S., Knaden, M., & Hansson, B. S. (2018). Spatial Representation of Feeding and Oviposition Odors in the Brain of a Hawkmoth. *Cell reports*, *22*(9), 2482-2492.

Brandt, K., Dötterl, S., Francke, W., Ayasse, M., & Milet-Pinheiro, P. (2017). Flower Visitors of Campanula: Are Oligoleges More Sensitive to Host-Specific Floral Scents Than Polyleges?. *Journal of chemical ecology*, *43*(1), 4-12.

Bruce, T. J., Wadhams, L. J., & Woodcock, C. M. (2005). Insect host location: a volatile situation. *Trends in plant science*, *10*(6), 269-274.

Burger, H., Dötterl, S., & Ayasse, M. (2010). Host-plant finding and recognition by visual and olfactory floral cues in an oligolectic bee. *Functional Ecology*, *24*(6), 1234-1240.

Bhagavan, S., & Smith, B. H. (1997). Olfactory conditioning in the honey bee, Apis mellifera: effects of odor intensity. *Physiology & Behavior*, *61*(1), 107-117

Daly, K. C., & Smith, B. H. (2000). Associative olfactory learning in the moth Manduca sexta. *Journal of Experimental Biology*, *203*(13), 2025-2038.

Daly, K. C., Durtschi, M. L., & Smith, B. H. (2001). Olfactory-based discrimination learning in the moth, Manduca sexta. *Journal of Insect Physiology*, *47*(4-5), 375-384.
Deisig, N., Sandoz, J. C., Giurfa, M., & Lachnit, H. (2007). The trial-spacing effect in olfactory patterning discriminations in honeybees. *Behavioural brain research*, *176*(2), 314-322.
Destaillats, H., Singer, B. C., Coleman, B. K., Lunden, M. M., Hodgson, A. T., Weschler, C. J., & Nazaroff, W. W. (2005). Secondary pollutants from cleaning products and air fresheners in the presence of ozone. *Proceedings of Indoor Air*, 2081-2085.
Desurmont, G. A., Laplanche, D., Schiestl, F. P., & Turlings, T. C. (2015). Floral volatiles interfere with plant attraction of parasitoids: ontogeny-dependent infochemical dynamics in Brassica rapa. *BMC ecology*, *15*(1), 17.
Dötterl, S., Vater, M., Rupp, T., & Held, A. (2016). Ozone differentially affects perception of plant volatiles in western honey bees. *Journal of chemical ecology*, *42*(6), 486-489.
Dudareva, N., & Pichersky, E. (2000). Biochemical and molecular genetic aspects of floral scents. *Plant physiology*, *122*(3), 627-634.
Farré-Armengol, G., Peñuelas, J., Li, T., Yli-Pirilä, P., Filella, I., Llusia, J., & Blande, J. D. (2016). Ozone degrades floral scent and reduces pollinator attraction to flowers. *New Phytologist*, *209*(1), 152-160.
Fiore, A. M., Jacob, D. J., Bey, I., Yantosca, R. M., Field, B. D., Fusco, A. C., & Wilkinson, J. G. (2002). Background ozone over the United States in summer: Origin, trend, and contribution to pollution episodes. *Journal of Geophysical Research: Atmospheres*, *107*(D15).
Finelli, C. M., Pentcheff, N. D., Zimmer, R. K., & Wethey, D. S. (2000). Physical constraints on ecological processes: a field test of odor-mediated foraging. *Ecology*, *81*(3), 784-797.
Fuentes, J. D., T'ai, H. R., & Zenker, J. (2013). Ozone impedes the ability of a herbivore to find its host. *Environmental Research Letters*, *8*(1), 014048.
Fuentes, J. D., Chamecki, M., Roulston, T. A., Chen, B., & Pratt, K. R. (2016). Air pollutants degrade floral scents and increase insect foraging times. *Atmospheric Environment*, *141*, 361-374.
Goyret, J., Markwell, P. M., & Raguso, R. A. (2007). The effect of decoupling olfactory and visual stimuli on the foraging behavior of Manduca sexta. *Journal of Experimental Biology*, *210*(8), 1398-1405.
Hallmann CA, Sorg M, Jongejans E, Siepel H, Hofland N, Schwan H, et al. More than 75 percent decline over 27 years in total flying insect biomass in protected areas. Plos One. 2017;12(10).
Hauglustaine, D. A., and Guy P. Brasseur. "Evolution of tropospheric ozone under anthropogenic activities and associated radiative forcing of climate." *Journal of Geophysical Research: Atmospheres* 106.D23 (2001): 32337-32360.
Haverkamp A, Bing J, Badeke E, Hansson BS, Knaden M. Innate olfactory preferences for flowers matching proboscis length ensure optimal energy gain in a hawkmoth. Nature Communications. 2016;7:9
Hauglustaine, D. A., & Brasseur, G. P. (2001). Evolution of tropospheric ozone under anthropogenic activities and associated radiative forcing of climate. Journal of Geophysical Research: Atmospheres, 106(D23), 32337-32360.
Hussaini, S. A., Komischke, B., Menzel, R., & Lachnit, H. (2007). Forward and backward second-order Pavlovian conditioning in honeybees. *Learning & Memory*, *14*(10), 678-683.

Kaissling, K. E., Hildebrand, J. G., & Tumlinson, J. H. (1989). Pheromone receptor cells in the male moth Manduca sexta. *Archives of Insect Biochemistry and Physiology*, *10*(4), 273-279.

Kevan, P. G. (1999). Pollinators as bioindicators of the state of the environment: species, activity and diversity. In *Invertebrate Biodiversity as Bioindicators of Sustainable Landscapes* (pp. 373-393).

Klein AM, Vaissiere BE, Cane JH, Steffan-Dewenter I, Cunningham SA, Kremen C, et al. Importance of pollinators in changing landscapes for world crops. Proceedings of the Royal Society B-Biological Sciences. 2007;274(1608):303-13.

Kluser, S., & Peduzzi, P. (2007). Global pollinator decline: a literature review.

Knudsen, J. T., Eriksson, R., Gershenzon, J., & Ståhl, B. (2006). Diversity and distribution of floral scent. *The botanical review*, *72*(1), 1.

Laloi, D., Bailez, O., Blight, M. M., Roger, B., Pham-Delègue, M. H., & Wadhams, L. J. (2000). Recognition of complex odors by restrained and free-flying honeybees, Apis mellifera. *Journal of chemical ecology*, *26*(10), 2307-2319

Leonard, R. J., Vergoz, V., Proschogo, N., McArthur, C., & Hochuli, D. F. (2018). Petrol exhaust pollution impairs honey bee learning and memory. *Oikos*.

Li, T., Blande, J. D., & Holopainen, J. K. (2016). Atmospheric transformation of plant volatiles disrupts host plant finding. *Scientific Reports*, *6*, 33851.

Lusebrink, I., Girling, R. D., Farthing, E., Newman, T. A., Jackson, C. W., & Poppy, G. M. (2015). The effects of diesel exhaust pollution on floral volatiles and the consequences for honey bee olfaction. *Journal of chemical ecology*, *41*(10), 904-91

Majetic, C. J., Raguso, R. A., & Ashman, T. L. (2009). Sources of floral scent variation: can environment define floral scent phenotype?. *Plant Signaling & Behavior*, *4*(2), 129-131.

McFrederick, Q. S., Kathilankal, J. C., & Fuentes, J. D. (2008). Air pollution modifies floral scent trails. *Atmospheric Environment*, *42*(10), 2336-2348.

Milet-Pinheiro, P., Ayasse, M., Schlindwein, C., Dobson, H. E., & Dötterl, S. (2012). Host location by visual and olfactory floral cues in an oligolectic bee: innate and learned behavior. *Behavioral Ecology*, *23*(3), 531-538.

Mota, T., & Giurfa, M. (2010). Multiple reversal olfactory learning in honeybees. *Frontiers in behavioral neuroscience*, *4*, 48.

Muhlemann, J. K., Klempien, A., & Dudareva, N. (2014). Floral volatiles: from biosynthesis to function. *Plant, cell & environment*, *37*(8), 1936-1949.

Murlis, J., Elkinton, J. S., & Carde, R. T. (1992). Odor plumes and how insects use them. *Annual review of entomology*, *37*(1), 505-532.

Pelz, C. O. R. I. N. N. A., Gerber, B., & Menzel, R. (1997). Odorant intensity as a determinant for olfactory conditioning in honeybees: roles in discrimination, overshadowing and memory consolidation. Journal of Experimental Biology, 200(4), 837-847.

Raguso, R. A., Levin, R. A., Foose, S. E., Holmberg, M. W., & McDade, L. A. (2003). Fragrance chemistry, nocturnal rhythms and pollination "syndromes" in Nicotiana. *Phytochemistry*, *63*(3), 265-284.

Reinhard, J., Sinclair, M., Srinivasan, M. V., & Claudianos, C. (2010). Honeybees learn odour mixtures via a selection of key odorants. *PloS one*, *5*(2), e9110.

Riffell JA, Lei H, Christensen TA, Hildebrand JG. Characterization and coding of behaviorally significant odor mixtures. Curr Biol. 2009;19(4):335-40.

Riffell, J. A., Lei, H., Abrell, L., & Hildebrand, J. G. (2013). Neural basis of a pollinator's buffet: olfactory specialization and learning in Manduca sexta. *Science*, *339*(6116), 200-204.

Schäffler, I., Steiner, K. E., Haid, M., Van Berkel, S. S., Gerlach, G., Johnson, S. D., ... & Dötterl, S. (2015). Diacetin, a reliable cue and private communication channel in a specialized pollination system. *Scientific reports*, *5*, 12779.

Shields, V. D. C., & Hildebrand, J. G. (2001). Responses of a population of antennal olfactory receptor cells in the female moth Manduca sexta to plant-associated volatile organic compounds. *Journal of Comparative Physiology A*, *186*(12), 1135-1151.

Theis, N., Lerdau, M., & Raguso, R. A. (2007). The challenge of attracting pollinators while evading floral herbivores: patterns of fragrance emission in Cirsium arvense and Cirsium repandum (Asteraceae). *International Journal of Plant Sciences*, *168*(5), 587-601.

Vingarzan, R. (2004). A review of surface ozone background levels and trends. *Atmospheric environment*, *38*(21), 3431-3442.

Visser, J. H. (1986). Host odor perception in phytophagous insects. *Annual review of entomology*, *31*(1), 121-144.

Wright, G. A., & Schiestl, F. P. (2009). The evolution of floral scent: the influence of olfactory learning by insect pollinators on the honest signalling of floral rewards. *Functional Ecology*, *23*(5), 841-851.

Supplementary Materials

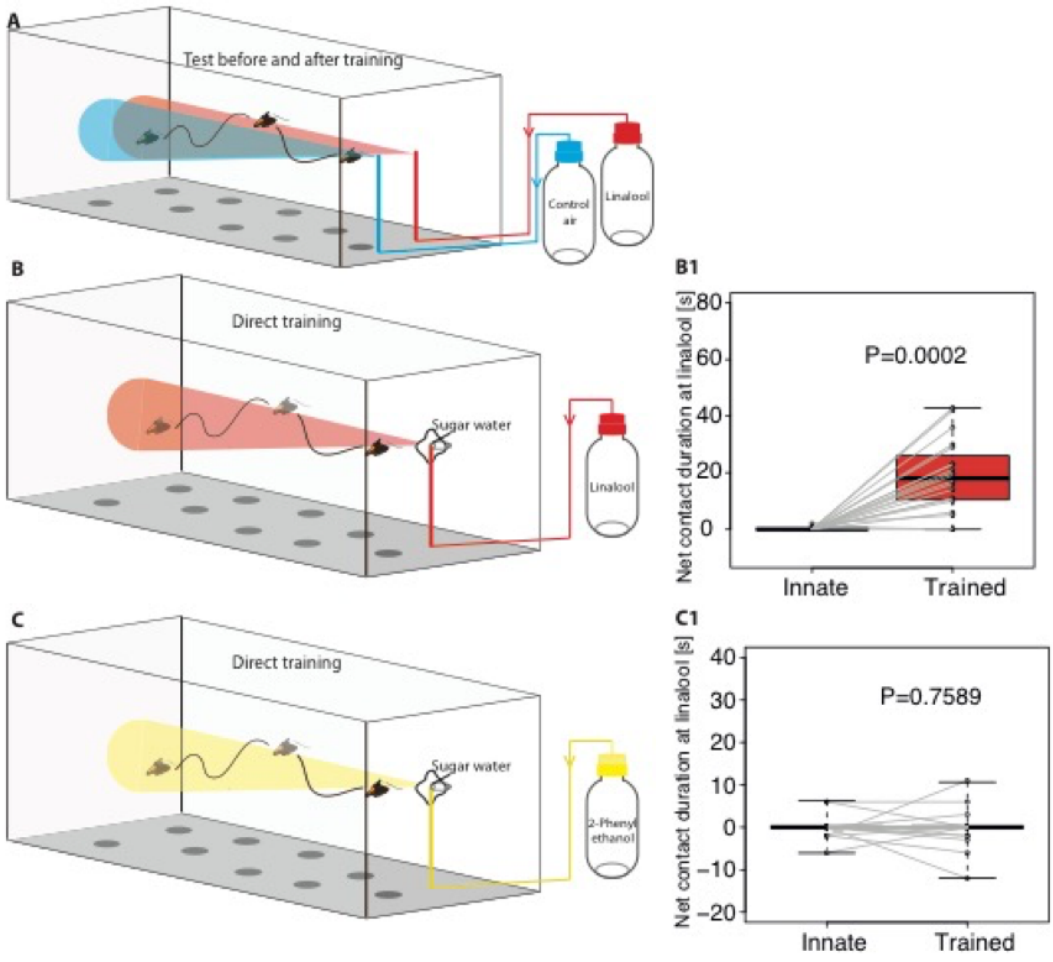

Fig S1. Moths can learn to associate an odor with sugar reward. **A.** Test situation before and after training sessions. **B.** Training, where moths are rewarded with sugar water at an artificial flower emitting a Linalool plume. **B1.** Net contact duration at Linalool plume before and after training (Net contact duration; time at Linalool plume minus time at control air source [s]) (Wilcoxon signed rank test, N=20). **C.** Training, where moths are rewarded with sugar water at an artificial flower emitting a 2-Phenyl ethanol plume. **C1.** Left boxplots, net contact duration at Linalool plume before and after training with 2-Phenyl ethanol (N=10).

Chapter #2

Effects of a tropospheric oxidant on sex pheromone mediated mate-location for the hawkmoth Manduca sexta

Summary

In many animal species, mate location is mediated by sex-pheromones, but sex pheromone communication may be vulnerble to interference from atmospheric pollution. Sex pheromone communication occurs via a three step process: pheromones are emitted by one partner, then travel some distance from the calling partner--through the atmosphere for land-based organisms--and finally are detected and interpreted by a conspecific. This system may be jeopardized by atmospheric pollution. Anthropogenically elevated atmospheric pollutants, namely tropospheric oxidants, could interfere with sex-pheromone signaling at all three phases: pheromone emission, transmission, and detection. We find that for the moth *Manduca sexta,* neither pheromone emission nor detection of olfactory cues on insect antennae is disrupted by elevated levels of the tropospheric oxidant ozone. However, pheromones in transit are disrupted by ozone: some components of *M. sexta's* pheromone plume are reactive with ozone, and ozone-exposed pheromone plumes are less attractive to mate-searching male *M. sexta. Manduca sexta* seem to perceive these ozone-altered plumes more faintly in the antennal lobe, the first-order processing unit of the brain. These findings highlight that pheromone plume transmission may be the most vulnerable aspect of sex pheromone communication to oxidants, and provides evidence that increased oxidants could interfere with mate location and contribute to the anthropogenic stressors driving recent declines in insect populations.

INTRODUCTION

Sexual reproduction is a common evolutionary strategy utilized by plants, fungi, protists, and animals. However, in order to participate in sexual reproduction, organisms must first overcome the often substantial obstacle of locating a mate. In mobile organisms, mate-location can involve long-distance signaling, where an organism produces a signal to attract a mate at some distance. To be an effective sexual signal, the signal must communicate to a conspecific the identity of the calling organism, its mate readiness, and its location. Calls to attract mates occur in a range of different modalities, including auditory signals such as birdsongs, (e.g. Date et al. 1991), visual signals such as luminescent firefly flashes (Stanger-Hall et al. 2015), and olfactory signals such as sex pheromones. While sex pheromones have been documented in many taxonomically diverse species ranging from elephants (Rasmussen et al. 1997) to lobsters (Atema et al. 1971), they are best characterized in moths, where the sex pheromones of over 530 species of moths have currently been identified (Ando et al. 2004). In moth species, females are typically the calling party. To attract a mate, a female moth will extrude her sex pheromone gland (Karlson and Butentandt 1959) and emit a single pheromone compound or blend of pheromone compounds that make up her distinctive sex pheromone plume. This pheromone plume then travels downwind where it can be detected and recognized as a mate-call by a conspecific male, who then has the opportunity to follow the sex pheromone plume, potentially over long distances, to the calling female (Greenfield 1981). For the many low-density or solitary moth species that search for mates without auspicious nighttime-specific visual cues (such as firefly flashes), olfactory cues may be critical to mate location and subsequent reproduction.

Sex pheromone plumes specificity and identification

In order to function as mate-signals, sex pheromones must act not only as long-distance cues, but as species-specific identifiers. Sex pheromone specificity is maintained through a robust mediation of both emission and reception of blends of pheromone compounds. The simplest form of sex-pheromone communication is one where a single compound is sufficient to attract mates. An example of a single-compound pheromone plume is bombykol. Emitted by the female silkmoth *Bombyx mori,* bombykol was the first identified sex pheromone: bombykol was described in 1959 by researchers Karlson and Luscher, who collected pheromone from more than 500,000 female *B. mori* to obtain 12mg of bombykol for identification (Karlson and Luscher 1959). Once sex pheromones such as bombykol were identified and synthesized, it became possible to investigate how male moths detect and recognize pheromone plumes. Further work on male *B. mori* moths has found that these males have olfactory receptors (ORs) tuned specifically to the pheromone bombykol (Sakurai et al. 2004). Expressing many sex pheromone ORs is not unique to *B. mori:* a study on two heliothine moths found that 72-81% of all ORS on the antenna, the major site of olfactory reception, were used for sex pheromone detection (Baker et al. 2004), and similar estimates have been made for the number of sex pheromone receptors on *Manduca sexta* (Kaissling et al. 1989). Furthermore, unlike the majority of olfactory receptors which are activated by a range of chemical cues, ORs for sex pheromones are highly specific (Wanner et al. 2010; Baker et al. 2004). Even small changes to the structures of sex pheromone compounds-- such as changes to isomerization, carbon chain length, or double bond location, make them unable to bind to sex pheromone ORs (Ando et al. 2004). When a pheromone compound does bind to an OR, that OR depolarizes and sends an electrical impulse from the antenna to the site of first-order processing in the insect's brain--the antennal lobe (AL) (Carlsson and Hansson 2006; Hansson et al. 2003). The first study of olfactory integration in the antennal lobe was performed on *Manduca sexta* (Matsumoto and Hildebrand 1981), and further

work determined that *Manduca sexta* pheromone compounds create a unique activation pattern in bundles of nerve-endings (termed glomeruli) in the AL, activating a region of the AL subsequently called the Macroglomerular Complex (MGC) (Christensen and Hildebrand 1987). The MGC is specific to male moths, and at least two *M. sexta* pheromone compounds, bombykal (E10,Z12-hexadcadienal) and EEZ (E10, E12, Z12-hexadacatrienal) (Tumlinson et al. 1989) integrate extensive dendritic branches across two or three specific glomeruli constituting the MGC (Hansson et al. 1991; Heinbockel et al. 1998).

While a sex pheromone 'plume' consisting of single compound is sufficient to induce effective mate-location in *B. mori*, such a simple one-pheromone system is rare among the more than 530 moth pheromone plumes currently identified (Allison and Carde 2016). More often, moth pheromone plumes are comprised of blends of pheromone compounds, and mate-searching males discern the identity of the female based on the composition of the pheromone blend. Male moths can recognize pheromone plumes of conspecifics based on the presence or absence of one or more pheromone compounds, the combinations of different compounds, the relative abundance of compounds, and the specific ratios of compounds in a pheromone plume. Due to their shared evolutionary history and the conserved nature of sex pheromones (Miura et al. 2009) many moth species share some common pheromone components, and even highly taxonomically disparate moths can have overlapping pheromone plumes (Allison and Carde 2016). In order to distinguish between the plume of a conspecific and the plume of another species containing many of the same pheromone compounds, male moths have developed two key methods of recognizing the sex pheromone plumes of their conspecifics. Male moths find conspecific plumes attractive only when very specific pheromone plume criteria are met, and they avoid the plumes of other moth species by finding key compounds or ratios of compounds in the non-conspecific pheromone plume to be deterrent.

The highly specific criteria of sex pheromone plumes necessary to induce attraction in male moths has been documented in hundreds of studies since moth sex pheromones were first identified (Hillier and Baker 2016; Landolt et al. 2006; Raina et al. 1986; Kaae et al. 1973; Vickers 2002; Teal et al. 1984; Roelofs et al. 1974; Zhang et al. 2012; Lofstedt et al. 1991; Nesbitt et al. 1973). In the heliothine moth *Heliothis pholoxiphaga,* pheromone plumes must consist of exact ratios of pheromone compounds: attractive pheromone plumes must consist primarily of (Z)-11-hexadecenal, but containing relative to (Z)-11-hexadecenal just 0.6% (Z)-9-hexadecenal, and between 2-3% (Z)-11-hexadecenol. Even small deviations from these ratios can cause the plume to lose attraction: decreases of (Z)-11-hexedecenol to less than 2% causes the plume to be dramatically less attractive to *H. pholoxiphaga* (Raina et al. 1986; Kaae et al. 1973). This discrimination based on relative ratios of pheromone compounds can help moths distinguish between similar pheromone plumes from different species: in the closely related congeneric moths *Heliothis subflexa* and *Heliothis virescens,* pheromone plumes of both moths contain the same two major and four minor pheromone components. It is only by trace amounts of an additional three pheromone compounds in the plume of *H. subflexa ,*which are absent in the plume of *H. virescens*, that moths can distinguish between the plumes. *Heliothis subflexa* requires those trace compounds to initiate mate-searching, while *H. virescens* finds plumes with one or more of the trace compounds unattractive (Vickers 2002; Baker et al. 2004).

Male moths may not only find plumes with blends slightly dissimilar from their target blend to be unattractive, they may also be actively deterred by the addition of pheromone compounds common to the pheromone plumes of other species. In the case of *H. pholoxiphaga*, less than 2-3% (Z)-11-hexedecanol causes the sex pheromone plume to lose its attractiveness, but an increase of (Z)-11-hexadecenol to just 4% causes the pheromone plume to act as a deterrent to *H. pholoxiphaga*. The noctuid moth *Helicoverpa zea,* which has a wide foraging

range across North and South America, is deterred by plumes containing even small amounts of the pheromone compounds (Z)-11-hexedecenol or (Z)-11-hexedecenyl acetate, compounds not present in *H. zea's* pheromone plume but found in the pheromone plume of at least eight other heliothine moth species (Hillier and Baker 2016). As with *H. phloxophaga*, concentration as well as composition can determine whether a pheromone blend is attractive or deterrent for *H. zea*: while 1-3% of (Z)-9-tetradecenal is necessary for attraction of male *H. zea*, more than 3% makes the plume deterrent (Descoins et al. 1988). Some of the pheromone compounds that a female produces may even serve a primary purpose in deterring mate-searching males of other species (Symonds and Elgar 2008), which could explain why the pheromone plume that males use to locate females is typically a subset of the complete blend emitted by the females (Vickers et al. 2017). Thus, female pheromone emissions are regulated not only by successful mating, but by avoiding unsuccessful mating. Likewise, males find pheromone plumes of their conspecifics attractive, but also find similar plumes that lead to non-mate compatible or non-con-specific females to be unattractive or deterrent, and males can thus avoid wasting energy in following plumes that do not lead to mate-ready conspecifics. It is only through the tightly controlled emission and interpretation of sex pheromone blends that moths are able to produce viable long-distance, species specific mating signals.

Anthropogenic oxidants may interfere with sex pheromone emissions, sex pheromones in transit, and sex pheromone reception

Mate selection mediated through tightly controlled sex pheromone signaling and reception may be jeopardized by anthropogenic air pollution. One species of air pollutants that are particularly threatening to sex-pheromone communication are highly reactive tropospheric oxidants. Tropospheric oxidants are secondary pollutants formed in the atmosphere as a result of fossil fuel

combustion, and they have the potential to interfere with the three stages of pheromone communication--pheromone emission, transmission, and reception. Oxidants could damage insect tissue, resulting in interference with pheromone emission and antennal pheromone reception, or they could react with pheromone compounds as they move through the atmosphere, altering the sex pheromone plume composition or concentration, and thus potentially changing the sex pheromone plume's meaning to mate-searching males.

While tropospheric oxidants have been present in the atmosphere over the course of the evolution of moth sex pheromone communication, the atmospheric load of tropospheric oxidants has increased sharply following the industrial revolution (Young et al. 2013; Naik et al. 2013; Monks 2005). One tropospheric oxidant, ozone, has increased from low estimates of pre-industrial levels of 10-12 ppbv (Hauglustaine and Brasseur 2001) to current averages in North America of 20-45 ppbv (Vingarzan 2004), with spikes in North America as high as 120 ppbv during daytime summertime ozone events (Fiore 2002; Vingarzan 2004). While ozone levels peak during the day, it is still an active oxidant during the nighttime (Yáñez-Serrano et al 2018) and could react with pheromone plumes released in the evening. Moreover, ozone can serve as a conservative estimate for the nighttime dominant oxidant: nitrate radical (Monks 2005). While ozone and nitrate radical have different compound-specific reaction chemistry and thus result in different alterations to pheromone plumes, both oxidants are highly reactive with alkenes, and nitrate can additionally abstract O-H and C-H bonds (Atkinson et al. 1984). Ozone's potential reactivity with sex pheromones and resultant interference with mate location could predict the effects of overall nighttime elevated oxidant concnetrations on moth sex pheromone communication.

Ozone reaction with pheromone plumes during transmission

Given the highly specific nature of sex-pheromone plumes, tropospheric pollutants may cause strong disruption to pheromone-mediated communication if plumes react with ozone as they are transmitted through the atmosphere. Elevated tropospheric oxidants could react with some components of pheromone plumes as they move downwind, both decreasing the distance that a sex pheromone blend can travel intact through the atmosphere and altering the composition of the plume. At least one current study has found that ozone can indeed react with sex pheromones of the moth *Tortrix viridana* (Klumpp et al. 2000). Changes to plume composition could be induced when ozone reacts with some sex pheromone compounds, causing a decrease in the abundance of these reactive compounds but not unreactive compounds. As a result, the plume not only experiences a loss of some compounds, but also a shift in the relative abundance of some pheromone compounds. Given male moths' reliance on specific relative abundance of some pheromone compounds to recognize conspecifics, these compositional changes to the plume could impair their function as sex pheromones, and potentially misrepresent the identity of the calling female to the mate-searching male.

Direct effects of ozone on moths; interference with pheromone emission and detection

Emission

In addition to altering pheromone blends in transit, elevated oxidants, as strongly reactive compounds, could also interfere with pheromone emission. Because ozone can react with carbon-carbon double bonds, it is reactive with both lipids and amino acids (Heath et al 1994; Goldstein et al 1969) and has the potential to damage the soft tissue of female sex pheromone glands. Female insects of several species possess the ability to sense their surrounding atmosphere and incorporate this information when deciding whether or not to call in order to maximize efficacy of the call and/or to reduce personal risk. Decreasing barometric pressure

indicative of an impending storm induces the taxonomically disparate species, true armyworm moth *Pseudaletia unipuncta* and the potato aphid, *Macrosiphum euphorbiae* to decrease their calling behavior (Pellegrino et al. 2013). Additionally, several moth species have been shown to autodetect their own sex pheromones in the surrounding atmosphere, and adjust their calling behavior to desist from calling when the concentration of their own pheromones is high, and resume calling again only when the concentration of their own pheromones decreases, as in the case where a wind removes the pheromone plume and provides a more favorable environment for pheromone transmission (Stelinski et al. 2014). As with sensing barometric changes or chemosensory detection of their own sex pheromones, female moths may detect the presence of highly elevated oxidants in the atmosphere and desist from calling in such oxidant-enriched environments, either to conserve pheromones or to keep the pheromone gland safe inside the female's body until atmospheric conditions for calling improve.

Detection

Furthermore, tropospheric oxidants may also interfere with sex-pheromone communication by damaging male olfactory receptors used to detect important chemical cues including sex pheromones. In order to detect important olfactory cues such as sex pheromones, insects filter air with these compounds through pores on their antenna, where specific binding proteins shuttle the compounds to olfactory receptors. As an insect filters air with odorants or pheromones through its antenna, it may also involuntarily take up tropospheric oxidants. Once inside the antenna, tropospheric oxidants such as ozone may cascade into a further series of oxidants that may continue to react with DNA (Victorin 1992), carbohydrates, and fatty and amino acids (Schaich and Pryor 1980; Roehm et al. 1971); the building blocks that make up cell walls and binding proteins. Through this mechanism, tropospheric oxidants may damage olfactory receptors.

Because the sensitivity of an insect's detection of odors is proportional to the number of olfactory receptors for a specific olfactory cue, damaging sex pheromone ORs could increase the detection threshold for pheromones, and thus inhibit a moth's ability to detect sex pheromones at low concentrations.

Research Gap

Recent studies have investigated the potential of anthropogenically elevated tropospheric oxidants to react with floral plumes and thus interfere with insect host location (Li et al. 2016; Farre-Armengol et al. 2015; Cook et al. in prep; Fuentes et al. 2013). However, the potential for tropospheric oxidants to interfere with sex-pheromone mediated mate-location may have equally if not more dire consequences for insect populations. While studies have found that ozone can react with sex pheromones of one moth species (Klumpp et al. 2000) and that elevated ozone impairs the use of an aggregate pheromone in aphids (Mondor et al. 2004), no current studies have tested both the reactivity of a sex pheromone plume and the response of a mate-searching insect to the ozonated sex pheromone plume. Furthermore, no studies have ascertained if ozone-exposure has an impact on either calling behavior or olfactory cue detection in insects.

RESEARCH OBJECTIVES

To understand how elevated oxidants may impact pheromone-mediated communication in moths, I investigate how an oxidant affects the three fundamental phases of pheromone communication: emission, transmission, and reception. Using the hawkmoth *Manduca sexta* and the oxidant ozone, we ask the following five questions:

(1.) Does ozone react with *M. sexta* sex pheromones

(2.) If so, do changes to the composition of sex pheromone plumes impact their attractiveness to male *M. sexta* moths flying towards pheromones or calling females in a wind tunnel?

(3.) Is a key pheromone in the blend of *M. sexta* perceived differently in the moth's antennal lobe after ozone-exposure?

(4.) Do elevated ozone levels deter female *M. sexta* from calling?

(5.) Do elevated ozone levels damage olfactory receptors on insect antennae, interfering with olfactory cue detection?

METHODS

Study Organisms Rearing

Male and female *Manduca sexta* were raised in separate temperature and light controlled chambers (light:dark= 16:8, 70% relative humidity and 25° C during the light phase, and 60% relative humidity and 20 °C during the dark phase) at the Max Planck Institute for Chemical Ecology in Jena, Germany. The moths light:dark cycle was set so that the moths experienced nighttime conditions during the day, and were thus available for wind tunnel behavior tests during daytime hours.

Pheromone collection and pheromone plume ozonation for behavioral assays, chemical analysis, and antennal lobe experiments

Sex pheromones used in behavior assays were extracted from female sex pheromone glands. Female moths used for pheromone extraction were given the opportunity to feed on 30% sucrose solution for two days before their sex pheromone glands were collected. Six or more females aged 3-4 days were collected in individual paper bags from their darkened chamber 2-3 hours

after their scotophase. Females were put in a refrigerator for 10-15 minutes to slow their movements, after which time they were removed from the refrigerator, and their abdomens firmly stroked so that their sex pheromone gland extruded. The pheromone gland was clipped with a pair of scissors, and using tweezers, was dipped in 300ul of hexane for three minutes, with the snipped end being held just above the hexane to prevent hemolymph from dissolving into the hexane solution. After three female pheromone glands had been dipped into the hexane, the solution was set aside and the remaining three female pheromone glands were dipped into another 300ul of hexane.

Pheromone wash ozonation for behavior assays

The pheromone wash used in behavior assays was used within 1-2 days of its collection. All pheromone wash to be used for experiments on a given day was blended together. Then, 300µl of the pheromone wash was pipetted onto a ~12mm disk of filter paper. This filter paper was then placed in a 1l glass bottle, and air that had first been scrubbed through a charcoal filter flowed into the bottle via a teflon tube at a rate of of ~0.3l/min, so that the flow being pushed out of the bottle passed through a flow meter a rate of 0.25l/min. Alternatively, the sex pheromone wash was exposed to ozone: instead of adding scrubbed clean air to the 1l glass bottle with 300µl of the female pheromone wash, air enriched with ozone to 120 ppbv was added through a teflon tube at rate of ~0.3l/min. Ozone was generated using a Thermo Scientific Ozone Generator (Model 165 Thermo Scientific inc., Pittsburg PA) and ozone concentrations were measured using an ozone analyzer (Model 202, 2B Technologies INc., Boulder CO). The 0.25l/min flows of either the pheromone plume in air or the pheromone plume in ozone-enriched air were transported from the 1l glass bottles to the wind tunnel via teflon tubes.

Pheromone and ozonated pheromone collection for chemical analysis and AL experiments

Both extracted and synthetic pheromones were exposed to ozone and clean air and chemically analyzed to investigate the reactivity of ozone with pheromone compounds, and the impact on pheromone blend composition. For extracted pheromone analysis, 600µl of pheromone wash was placed in 250ml airtight glass bottles with a glass pipette superQ screwed into the lid. Either scrubbed air or air enriched with 120 ppbv ozone was pushed into the bottles at a rate of 0.25l/min, and 0.25l/min was vacuumed out via the top of the superQ, so that the pheromone plume from the bottle was forced through the SuperQ. The pheromone plume was collected on the superQ for either 2 or 12 hours, after which time, the superQ was removed from the bottle and was eluted with 500µl of hexane. The samples were then evaporated to 100µl using a low and steady flow of N_2 before being injected into the GC-MS. Nine samples of 600ul pheromone wash mixed with air and six samples of 600ul pheromone wash mixed with ozone were used for PCA and t-test analysis.

Due to the very low concentration of pheromones in the pheromone wash, synthetic pheromones previously identified from the blend of *M. sexta* were also used to assess the effect of ozone on pheromone structure and to investigate how ozone-exposed pheromones may be perceived differently in the antennal lobe of male moths. 100 µl of individual synthetic pheromones dissolved in hexane was pipetted onto a 12 mm filter paper disk and then exposed to either ozone or air using the same superQ collection described above for extracted sex pheromones. While the amount of pheromone pipetted on the filter paper was kept constant at 100 ul, two concentrations were tested: 10^{-3} and 10^{-2} of bombykal and (Z9)-hexadecenal and were collected at two time intervals: 2 and 12 hours. The (Z9)-hexedecenal but not the bombykal used in this study was stored in hexane solution with butylated hydroxytoluene, an antioxidant compound intended to stabilize (Z9)-hexedecenal in storage. Only the highest concentration of

pheromones collected over the shortest amount of time showed traces still containing the original pheromone, so these samples were used for further t-test and PCA analyses (Fig1B&C) Ten samples of 100 µl 10^{-2} bombykal were mixed with either air (five samples) or ozone (five samples) for two hours: these ozone exposed vs. air-exposed samples were then compared with PCA and t-test analyses. Similarly, six samples of 100ul of 10^{-2} (Z9)-hexedecenal mixed in air and five samples of 100ul of 10^{-2} (Z9)-hexedecenal mixed in ozone were used for PCA and t-test analyses. Additionally, sample traces of 100ul of 10^{-2} bombykal and (Z9)-hexedecenal exposed to ozone and air over a 12hour collection period were visually examined (Supplementary Figure1), leading to the decision to only analyze bombykal and (Z9)-hexedecenal collected at the highest concentration and the shortest collection time.

Because our analysis showed that bombykal was not stable even when exposed to just the clean air during SuperQ collection, we further examined the composition of bombykal by direct injection in the GCMS, and by collecting bombykal plume via SuperQ exposed only to argon. We collected bombykal in 0.25l/min argon, and 0.2l/min argon mixed with 0.05l/min ozone at ~200 ppbv, so that the mean concentration of ozone exiting the bottle was 120 ppbv. Ozone levels exiting the bottle were kept relatively constant at 120 ppbv by manual monitoring of the ozone concentration during the two-hour pheromone collection time. Five samples of bombykal mixed in argon, and five samples of bombykal mixed in argon with added ozone were collected and used for t-test and PCA analyses. In post-collection analysis of pheromone and pheromone breakdown products in air and in ozone, NIST library software was used to identify the breakdown products.

Male choice assays for pheromone wash exposed to air or ozone

Before male moths were used for choice assays, they were first allowed to forage on real and artificial flowers in the wind tunnel. One potted and flowering plant of *Nicotiana alata*, a favored host plant of *M. sexta*, was placed in the wind tunnel, along with four supplemental artificial flowers constructed from white filter paper disks with eppendorf tubes at the center containing 1ml of a 30% sucrose solution in DI water apiece. Males were released in the wind tunnel, and after they located the flowers, they were given at least two minutes to feed before being removed from the wind tunnel and placed in small individual mesh cages. Each male in a mesh cage was then given at least 45 minutes to rest in a small room adjacent to the wind tunnel with the same temperature, lighting, and humidity metrics as the wind tunnel.

After this feeding preparation, male moths were flown individually in the wind tunnel to assess their response to the pheromone plume in scrubbed clean air and the pheromone plume mixed with ozone-enriched air. At the downwind side of the wind tunnel, two metal poles held upright teflon tubes through which flowed the pheromone plume (300µl of the pheromone wash) mixed either with air or with ozone: both plumes were generated as described above. On each metal cylinder, a visual cue was used to further attract the males-- a brown paper triangle with a height of ~7.62cm was laid on its side, and a wedge was cut out of the base to simulate the shape of a stationary female. Individual males were given four minutes in the wind tunnel to investigate the paper 'female' emitting either the pheromone plume mixed with clean air, or the pheromone plume mixed with ozone-enriched air. Both first choice and time spent investigating the paper females were recorded: investigation time was counted if the male was hovering close to the paper flower or exhibiting signs of copulation attempts, such as abdomen curling. Occasionally, a male would extend his proboscis towards the paper female, and in such cases, the male was recorded as having no mate-searching response to the pheromone plume. Male preference for pheromone plumes mixed with ozone vs. air was assessed by comparing the number of initial

investigations (first choice) to ozone vs. air-exposed pheromone plumes with a Chi-square test. Further, the amount of time that males spent at paper females emitting the ozone-exposed vs. air-exposed plumes was compared with a Wilcoxon signed rank test,as the data could not be transformed in a manner to meet assumptions of normality for a t-test.

Male choice assays for calling females

We also investigated the response of male *M. sexta* to calling females in the wind tunnel with a flow of 120 ppbv ozone or with a flow of air. To test their response, males in their individual baskets were placed in the room adjacent to the wind tunnel and given at least 1 hour to acclimate to the wind tunnel conditions. During this time, females that were three days old and had been given the opportunity to forage on 30% sucrose water were placed in a large mesh cage in the wind tunnel room lit only with red light, and observed. When a female began calling, she was collected from the large mesh cage and put into a small individual basket at the downwind side of the wind tunnel. Another, empty individual mesh cage was already placed at the downwind side of the wind tunnel to act as a control. Either clean air or ozone-enriched air of 120 ppbv was added to the wind tunnel in two plumes of 1l/min apiece. Plumes of air or ozone-enriched air entered the wind tunnel via teflon tubes, one affixed to the empty mesh cage, and the other affixed to the mesh cage with the calling female.

With this setup complete, individual males were placed at the downwind side of the wind tunnel and given four minutes to investigate the mesh cages. After four minutes of flight in the wind tunnel the males were removed and the female was assessed for calling status. If she was still calling, another male was tested in the wind tunnel. If she had stopped calling, the response of the male was not counted for the study. Male response to female calling was assessed with a

chi-square test comparing the number of first-choice investigations of calling females vs. the empty container in air vs. ozone-enriched conditions.

Examination of ozone-altered pheromones in the antennal lobe

Image 1. Antennal lobe preparation for *M. sexta*.

We next assessed response of male moth's antennal lobe (AL) to one of the key pheromone components of *M. sexta's* plume mixed with air vs. mixed with ozone. Males used for this study were restrained in a plastic tube, their proboscis was restrained with dental wax, the scales were removed from their skull, and a window was cut into the skull to reveal the insect brain (Image 1). The top mass of the brain was carefully removed with tweezers under the microscope, until the antennal lobe was visible and unobstructed from above (Image.1). A calcium-sensitive dye (CaGr-2-AM) in a mixture of 80% *Manduca sexta* ringer and 20% Pluronic F-12 DMSO (Molecular Probes, Eugene, Ore., USA) was applied to the AL, filling the skull cavity. Males were then placed in a small box with a damp paper towel to prevent the antennal lobe tissue from drying out, and placed in the fridge for 12 hours to allow the agitated AL to relax and provide a clearer signal-to-noise ratio during odor testing.

Prepared moths were then tested for their response to a puff of the single pheromone component bombykal exposed to air vs. bombykal that had previously been exposed to ozone-enriched air at a concentration of 120ppbv. Pheromone plumes exposed to air vs. to ozone were generated by placing 100ul of 10^{-2} bombykal on filter paper in a 1l bottle collected on superQ for 2 hours, with a flow of 0.5l/min flow of either scrubbed clean air or 120ppbv ozone. The superQ

was eluted with 500ul of hexane and blown-down with N_2 to 100ul.To control for the response of the AL to the hexane solvent, moths were also tested for their response to pure hexane. All odors were presented to the moth in a moistened, charcoal-filtered continuous airstream at rate of 30ml/s. Odors were presented directly in front of the moth via a small glass tube, which had a small inlet in which the tip of the pipette which connected to the airflow system and contained 100ul of the sample (either Bombykal + Ozone in hexane, Bombykal +Air in hexane, or hexane) on filter paper was inserted. A simultaneous flow controller (syntech) established a constant flow so that the moth antenna was always stimulated by 3ml/second of airflow, and an instantaneous switch to the flow through the pipette puffed the odor at the moth for 500ms. Moths were placed under an upright microscope (Olympus) and activation of regions of the brain, as assessed by fluorescence of calcium in the calcium-sensitive dye, was recorded using a TILL photonics imaging system (Fig 2B).

Female calling behavior

To determine if the presence of elevated ozone affected female calling, calling females were exposed to a plume of either ozone-enriched air or clean air, and the amount of time spent calling over the next five minutes was measured. A single three-day old female *M. sexta* was put in a small empty pipette tip box--a small inlet was drilled into the box and a teflon tube that was connected to the box and then to either a pump emitting clean air, or an ozone generator, and the connection between the tube and the box were sealed with silicone wax. The box with the female was then placed directly under a red-light camera in a room lit only with red light. A flow of either scrubbed clean air or ozone-enriched air flowed into the box at a rate of 0.5 l/min so that a constant flow of air or ozone-enriched air passed over the moth. Typically within a minute of the flow being turned on, the female *M. sexta* would begin to call. Once the moth began calling--

which was visually assessed via the video captured on the red-light camera projected on a computer outside of the dark room--a timer was started, and the total amount of time the moth spent calling in the next five minutes was recorded.

To ascertain realistic and potential effects of ozone on the calling behavior of *M. sexta* calling, females were exposed to three different air pollution treatments: either scrubbed clean air, air enriched to 120 ppbv ozone--a high but realistic ozone concentration for rural North America--or 500 ppbv ozone, an extremely elevated concentration of ozone that has been attained in major cities in the US before EPA air quality regulations were imposed (Parrish et al. 2011).

The effect of ozone on female calling behavior was assessed using a one-way analysis of variance to compare the total amount of the test time that females spent calling under the different ozone concentrations of no additional ozone, 120ppbv ozone, and 500ppbv ozone.

Effects of ozone on antennal sensitivity to olfactory stimulus

Male moth exposure to ozone or air

Ozone may damage insect olfactory receptors and decrease the sensitivity of an insect antennae to olfactory cues such as sex pheromones. To separate the impact of ozone on insect antenna and potential impact of ozone on chemical cues, both of which could affect the insect's perception of a cue, we first exposed moths to elevated ozone and then tested their antennal response to compounds presented only in clean air. To examine the impact of longer-term ozone exposure to antennal sensitivity, one-day old naive *M. sexta* were exposed for three 8-hour periods to either scrubbed clean air or ozone at a concentration of 120 ppbv. For ozone exposure treatments, two males at a time were placed in one of two cylindrical glass chambers, where one glass cylinder had a flow of 2l/min of scrubbed clean air flowing continuously for three days, and the other had

a 2l/min flow of ozone at 120 ppbv for eight hours a day (between the hours of 8am and 10pm), with scrubbed clean air flowing through the cylinder for the other 16 hours a day. Cylinders used for ozone vs. air treatments were switched between every set of moths. After three days of either the air or ozone-enriched air treatment, the males were removed from the cylinder and tested for their antennal sensitivity to odors.

Scent preparation and EAG

Given the high percentage of ORs tuned to pheromone compounds, we sought to assess damage to moth antennae by testing the impacts of ozone-exposure on less abundant ORs, where a decrease in function of just a few hundred receptors could constitute a strong decline in antennal response. Three odors at five different concentrations were selected using an electroantennogram (EAG).Three odors, linalool, methyl salicylate, and cis-3-hexenyl acetate were selected for this study based on their different chemical structures and the existence of previous reports of EAG response to these odors for male *M. sexta* (Fraser et al. 2003). All three compounds, purchased from Sigma Aldrich had a purity 95-99% and were prepared at five concentrations ranging from 10^{-5}- 10^{-1} using mineral oil as the solvent. 10µl of each mixture was pipetted onto a circle of filter paper and then inserted with tweezers into a glass Pasteur pipette. A small amount of paraffin wax was placed at the bottom of the pipette and a plastic pipette tip was placed at the top to seal the pipette until it was used in EAG recordings. Samples of pure mineral oil were also prepared in this fashion to act as a control for the odor stimulus.

After either exposure to ozone or to air treatments, a male antenna was cut off at the base and attached to a metal fork with electrode gel (Spectra 360 ElectroGel, Parker ce).This fork was then attached to an EAG located on an air-stabilized anti-vibration table. A continuous flow of

scrubbed clean and humidified air was passed over the antenna, after passing through a simultaneous flow controller.

To present the stimulus to the moth, the pipette tip of an individual odor sample was fitted into another pipette tip attached to a plastic tube which was connected to a stimulus controller (Syntech Model CS 55) , which kept the airflow on the insect antenna constant when it was switched between clean air and air puffed through the odor-prepared pipette. A foot pedal was used to activate the stimulus controller, so that the flow of scrubbed air switched from clean air to puffing 5-cc of air over 200msec through the Pasteur pipette. The odors were removed from the area of the insect antenna after this 200msec pulse as the flow of clean air automatically returned. The response of the antenna to the odor stimulus was measured using an EAG Syntech INR-2 and analyzed using Syntech software. All odors were presented in clean air: no ozone was used in EAG tests.

Each *M. sexta* antenna from air-exposed and ozone-exposed moths was tested against the three scents at the lowest concentration, and then tested for their EAG response to just mineral oil before moving on to test them with the three scents at the next highest concentration. Each scent at each concentration was puffed three times to ensure consistency of the plume from the pipette, and the puff of only mineral oil was tested as a control after each set of three puffs. Thus, each *M. sexta* antenna had a total of 54 recordings. Over the course of EAG recording, some moth antennae expired before the final sets could be conducted, and moths that showed a decrease in response (after accounting for response to mineral oil) to an odor that they had previously responded to were removed from the study. A total of eight moth antenna were used for data analysis, with four being removed due to lack of responses. Due to errors in the EAG recording software and moth mortality, it was not possible to consistently test an ozone vs. air treated moth from the same two-day exposure set.

RESULTS

Sex pheromone analysis

In the sex pheromone wash, it was possible to discern a decrease in abundance for some specific pheromone compounds (Bombykal and (Z)-7-Hexadecenal) in ozonated plumes when comparing a single pheromone wash blend mixed either with ozone or with air. However, the high variability in pheromone plume washes, it was not possible to determine if the composition or overall abundance of total volatiles in pheromone wash plumes were altered by ozonation.

Tests of single pheromone compounds collected over the longer period of 12 hours (rather than for 2hours) or at the lower concentrations of 10^{-3} (rather than concentrations of 10^{-2}) did not show consistent presence of the single pheromone for bombykal in air or in ozone, or consistent presence of (Z)-9-hexadecenal in ozone (Supplementary Fig 1). This was because the concentration of 10^{-3} was too low, and because exposing the pheromones to air or ozone for 12 hours caused a high degree of degradation: in both cases, the result was an absence of the pheromone compound from some traces at these lower concentration, longer collection time tests. Therefore, samples of single compounds at the highest concentration of 10^{-2} collected over 2 hrs were used for further analysis of single pheromone compounds exposed to either ozone or air. Analysis of higher concentration and shorter collection time samples for the single synthetic compounds bombykal and (Z)-9-hexadecenal find that ozone significantly reduced the abundance of (Z)-9-hexadecenal relative to abundance of the compound in air, but did not significantly reduce the abundance of bombykal. The lower abundance of (Z)-9-hexadecenal in ozone-exposed vs. air-exposed traces demonstrates that (Z)-9-hexadecenal reacts with ozone. Further supporting this idea, while breakdown compounds are present in both air and ozone-exposed (Z)-9-hexadecenal plumes, the plume composition was different in air and ozone-exposed plumes, as they were distinctly separate as assessed via PCA. Bombykal mixed with air

and mixed with ozone are also separate via PCA, although bombykal concentration is not significantly reduced after exposure to ozone, which may be due to high variability of bombykal concentration collected in clean air, which ranged in abundance from 0E+00-3.85E+07.

The high number of breakdown products in the bombykal plume mixed only with clean air made it very challenging to compare between this supposed 'control' and the plume of the ozone-mixed compound. To clarify where the breakdown compounds were being formed in the air-mixed plume, we further directly injected 10^{-3} bombykal in hexane into the GC-MS (Supplementary Fig 1) and collected bombykal in argon. In this argon-exposed scenario, bombykal was found to be significantly decreased after exposure to ozone relative to exposure to air, and bombykal mixed with ozone showed breakdown compounds not present in the only argon-exposed trace.

Comparison of traces for ozone-exposed and air-exposed sex pheromones.

Sample traces of pheromones and PCA are shown in Figure 1, and compound identification is found in Table 1. The compounds could only be identified with NIST software to >75% certainty, and pure compounds collected on SuperQ were run with their standards.

Sex pheromone wash was collected for 12 hrs of exposure to air or ozone. For the pheromone wash, there was no clear distinction in air-exposed or ozone-exposed traces via PCA analysis, and the total abundance of pheromone wash in air and in ozone were not significantly different (Fig 1A t-test: p-value = 0.413, n=16).

10^{-2} Z9-hexedecenal exposed to ozone and air for the same 2hrs collection time showed visually separate traces as assessed by visual differences in samples plumes and separation in PCA, and (Z9)-hexadecenal was reduced in ozone-exposed traces relative to air-exposed traces (Fig 1B: t-test: t=2.421, p-value = 0.043, n=11)

Bombykal in air was not significantly decreased with exposure to ozone vs. to air (t-test: t=1.85 p-value = 0.113, n=10) even though the structure of the plume looks different from PCA analysis and from samples traces (Fig 1C). Bombykal in argon mixed with ozone however was significantly decreased from bombykal without ozone (Fig 1D: t-test: t= 2.862, p-value = 0.0322, n=10).

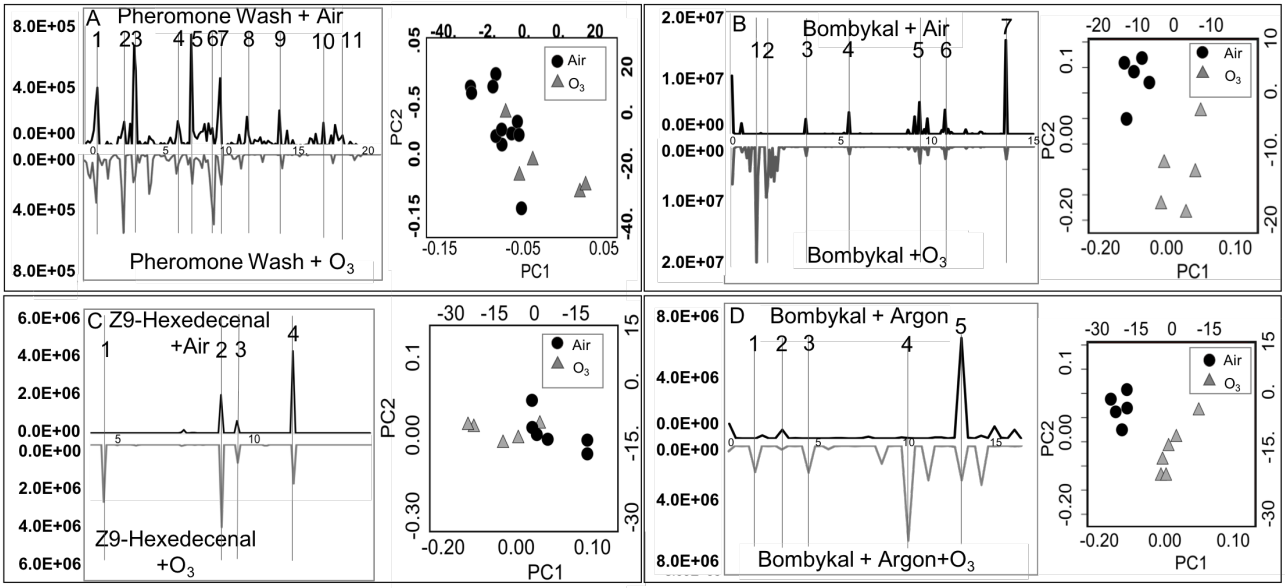

Fig. 1. Sex pheromones reacting with ozone: black traces are a sample trace of pheromones mixed with air, and grey traces represent pheromones mixed with 120ppbv ozone. **A.** Air and ozone-exposed traces of sex pheromone differed visually, but PCA showed no clear separation. **B**. sample ozonated and unozonated traces of Z9-hexedecenal were visually and distinct showed separation in PCA **C.** Sample trace of ozonated and unozonated bombykal in air are visually distinct and show separation via PCA. **D.** Sample trace of Bombykal in argon appeared different and PCA were distinctly separate.

Table 1.

Sex pheromone and compounds Identification

	Sex Pheromone Wash
1	2-ethyl 1-hexanol
2	Nonanal
3	3-Decanone
4	Si
5	Decanal
6	Tetradecane
7	Z-7-Hexadecenal
8	2-ethylexyl salicylate
9	Bombykal
10	Si
11	Heneicosane

	Bombykal in Air
1	Octenal
2	Si
3	Nonanal
4	Decanal
5	10-Undecenal
6	11-tridecyn-1-ol
7	Bombykal

	Z9-Hexedecenal
1	Octenal
2	Decanal
3	Butylated hydroxytoluene (added antioxidant)
4	Z9-Hexedecenal

	Bombykal in Argon
1	Nonanal
2	Si
3	Decanal
4	10-Undecenal
5	Bombykal

Table 1. Numbered peaks from sex pheromone wash in air vs. ozone, bombykal in air vs. ozone, (Z)-9-hexadecenal in air vs. ozone, and bombykal in argon vs. bombykal in argon mixed with 120 ppbv ozone are identified by the NIST library (R-match > 75%).

Male mate-searching in response to calling females and sex pheromones in ozone and air

Males tested for their response to calling females in ozone-enriched or clean air in the windtunnel did not show a significant preference for females in air. However, when males were tested for their response to ozone-exposed vs air-exposed plumes of sex pheromone wash, the males preferred the air-exposed plumes. The decrease in attractiveness of ozone-altered plumes is correlated with a decrease in magnitude of the AL response to ozone-exposed pheromone plumes.

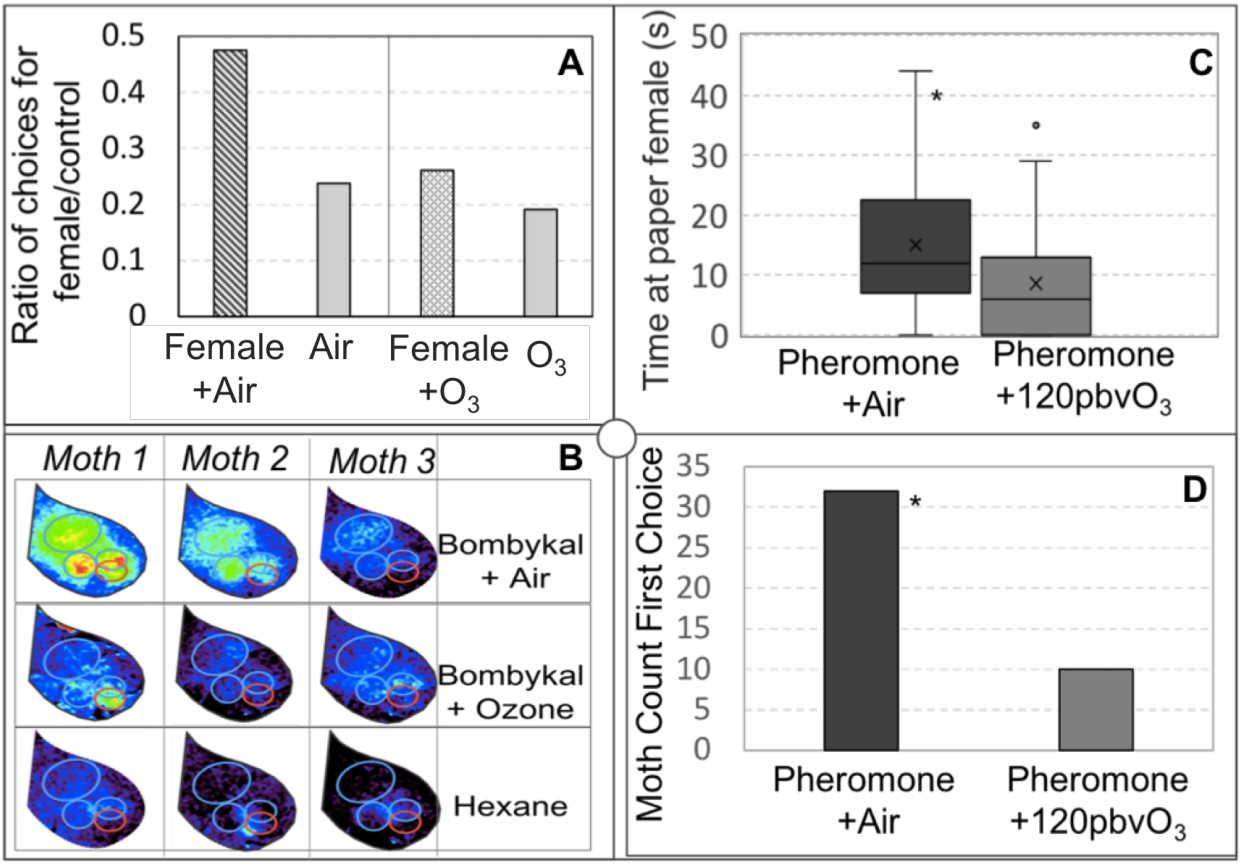

Fig. 2. Male response and perception of sex pheromones in ozone. A. Males did not approach females in clean air conditions more often than in ozone-enriched conditions (Female +Air vs. Air χ^2 =0.413 *p*-value=0.0747; Female +O3 vs. Air (χ^2 =0.041 The *p*-value=0.847). **B**. Bombykal exposed to ozone vs. air had an apparent decrease in activation of the antennal lobe for one glomerulus in the MGC (circled in red). **C.** Male moths spent more time investigating the sex pheromone plume presented in air relative to the time spent investigating the sex pheromone plume mixed with ozone (Wilcoxon test: W = 2659, *p*-value = 3.907e-05). **D.** Males first investigated the pheromone wash mixed with air relative to the pheromone wash mixed with ozone more often (χ^2 =3.7193, *p*-value = 0.000141)

The direct effects of ozone on female *M. sexta* pheromone emission and male *M. sexta* olfactory cue detection were minimal. Males exposed to ozone for an extended time only showed

a decrease in antennal response to one compound at one concentration, while increasing ozone levels had no discernible effect on the short-term calling behavior of female *M. sexta*

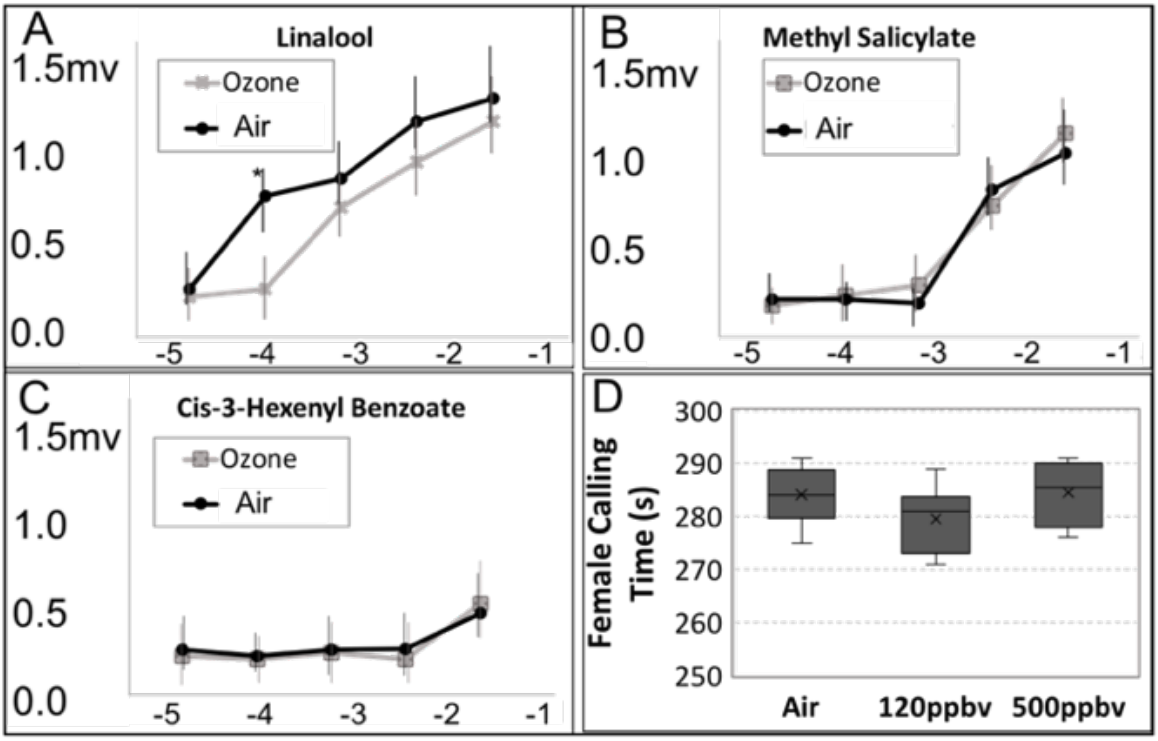

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Fig 3. **A-C:** Antenna of male moths exposed to ozone had a significantly reduced response to 10^{-4} linalool (t-test with bonferroni correction: t= -2.82, *p*-value= .0010) but not to any other odor at any other concentration. **D.** There was no difference in the amount of time that females spent calling in any of the three treatments of extremely high ozone (5,000 ppbv), high but realistic ozone (120 ppbv), and scrubbed clean air (~12 ppbv ozone) (ANOVA: F= 0.274, *p*-value =0.761)

DISCUSSION

Ozone alters pheromone compounds

Ozone can react with *M. sexta* sex pheromone compounds (Fig.1). Exposing the full pheromone wash to ozone decreased some pheromone compounds, as assessed when a given sex-pheromone wash was exposed to air or ozone. However, high variability in both abundance and composition of plumes of different pheromone washes obscured these differences when comparing all ozone and air-exposed plumes. Differences in pheromone washes may be caused by variation in female pheromone production, and may also be attributed to the presence of *M. sexta* tissue in some samples. Heneicosane was detected in 9 of the total 15 samples of the 600ul of *M. sexta* pheromone wash samples, and heneicosane is more likely attributed to the presence *M. sexta* tissue rather than *M. sexta* pheromones. The individual synthetic compounds bombykal and (Z)-9-hexadecenal, however, gave more clear profiles than the pheromone wash. Both bombykal and (Z)-9-hexadecenal were consistently affected by ozone, showing differences in concentration and composition after two hours of exposure to 120 ppbv ozone vs. scrubbed air (Fig.1). Surprisingly, even the synthetic pheromone compounds mixed with scrubbed air treatments showed a considerable amount of breakdown components, particularly for bombykal; (Z)-9-hexadecenal had many fewer breakdown products, which may be attributed to the presence of butylated hydroxytoluene, an added antioxidant, in the (Z)-9-hexadecenal solution. The breakdown seen for (Z)-9-hexadecenal and more prominently for bombykal exposed only to clean air may be caused by the presence of oxidants still remaining in the scrubbed air, and the unstable nature of the pheromone compounds. In a natural environment, pheromone compounds are not likely to be used as a cue for the length of time (2 hrs) that they were exposed to either air or ozone-enriched air in this experiment, so it should be noted that the breakdown of pheromone compounds in air, while useful for comparing to breakdown of compounds in ozone over the same time, may not be fully representative of a pheromone that could be encountered by a mate-searching male *Manduca sexta* moth.

To control for the effects of overly-lengthy exposure to air used for collecting pheromone compounds with SuperQ, we ran a further test collecting bombykal in either the unreactive noble gas argon, or in argon mixed with air enriched with 120 ppbv ozone. From these samples, we find that ozone significantly decreased the concentration of bombykal, but that the bombykal exposed only to argon still contained some breakdown components not present when the compound in solution was injected directly into the GC-MS (Supplementary Fig1). The samples may have been exposed to air as they were eluted from SuperQ, or the pheromone compound may decompose at room temperature when present in hexane. Our findings that both (Z)-9-hexadecenal and bombykal are decreased after reaction with ozone relative to either air (Fig.1B) (in the case of (Z)-9-hexadecenal) or argon (Fig.1D) (in the case of bombykal), and that the compounds in ozone show distinctly different traces than compounds in air, align well with previous findings that ozone reacts with some sex pheromone compounds of the green oak tortrix. In the case of sex pheromones of the green oak tortrix moth, ozone reduced the abundance of some pheromone compounds and caused the formation of secondary products that altered the plume composition (Klumpp et al. 2000).

Ozone caused pheromone plumes to be less attractive to male M. sexta

While our findings on the effect of ozone on sex pheromone plumes was mixed, with decreases of compounds found for individual compounds but high variability in the sex pheromone plume wash, the response of male *M. sexta* to ozone-exposed pheromone plumes was clear: pheromone wash exposed to ozone was less attractive to mate-searching males. Male *M. sexta* flying in the wind tunnel spent more time investigating paper ‘females’ exuding air-exposed pheromone wash compared to a paper female emitting the ozone-exposed pheromone wash (Fig.2C). Additionally, male moths initially visited paper ‘females’ that exuded the pheromone wash in clean air more

often than they visited the paper 'females' that exuded the pheromone wash mixed with ozone (Fig.2D). Male *M. sexta* were not deterred by ozone itself (see Cook et al. in review), so their preference for the sex pheromone mixed with air vs. mixed with ozone must be driven by ozone-induced changes to the sex pheromone plume. Both compositional and concentration changes were observed for bombykal and (Z)-9-hexadecenal exposed to ozone (Fig.1), and either change could have driven the loss of preference. Higher concentrations of sex pheromones may be indicative of larger and thus preferred females (Harari et al. 2011), which could drive male preference for higher-concentration, air exposed pheromone wash. The loss of attraction could also be due to the altered composition of the plume resulting in a plume that is less typical or totally atypical of *M. sexta's* pheromone plume.

Male moths expressed no significant preference for females in the wind tunnel with an influx of 120 ppbv ozone or with clean air (Fig.2A). The moth's discernment between the ozone and air-exposed calling females may be obscured by small sample sizes. These small sample sizes were driven by the unresponsiveness of the male moths to calling females even in the control: nearly 30% of male moths investigated the empty basket rather than the basket containing the calling female even in clean air conditions. The male *M. sexta's* lack of attraction to calling females my indicate that either female or male *M. sexta* used in the experiment were not healthy, and both male and female *M. sexta* colonies experienced sharp declines in population during the course of this experiment, although no cause for this decline could be discerned. The population of both male and female moths went to zero during the course of these experiments, cutting off the sample size below the desired number.

Ozonating bombykal may cause it to be perceived less strongly in the AL.

To better assess if the males' preference for pheromone plumes exposed to air vs. exposed to ozone was driven by changes in concentration or changes in composition of the pheromone plume, we assessed how a key pheromone compound was perceived in the antennal lobe of male *M. sexta.* Bombykal mixed with 120 ppbv ozone elicited a lower activation of the same glomeruli than bombykal mixed only with air. However, while some glomeruli in the Macroglomerular Complex (MGC) showed a decrease in response to the ozone-altered bombykal compound, the activation pattern was not apparently altered from the bombykal plume exposed to air, showing no additional glomeruli activation when exposed to ozonated bombykal (Fig2.B). The activation of glomeruli in the MGC for both ozone-altered and air-expose bombykal indicates that there was no change activation pattern in response to bombykal in air, but that there was a decrease in response to the ozone-exposed bombykal. If the mixture of bombykal, ozone, and the subsequent breakdown products resulting from ozone and bombykal's reaction do not change the AL activation pattern, it is likely that the breakdown products are either activating the same receptor as bombykal, or not activating any receptors, so that the moth perceives bombykal mixed with ozone as a decrease in bombykal concentration rather than as a different odor cue.

Ozone does not impact female calling behavior

While ozone reacted with pheromone plumes in transit, it had no discernable impact on female *M. sexta* calling behavior. Even at extremely elevated concentrations of 500 ppbv, ozone did not deter female moths from calling. While continued calling in the presence of high pollution may at first seem to offer resiliency in the face of perturbed atmospheres, females calling in polluted areas are emitting pheromones that, in accordance with our behavioral assays in the wind tunnel, are less attractive to males. Since oxidant concentrations are variable over hourly timescales,

females that could sense elevated oxidant loads and adjust their behaviors to avoid calling during peak oxidant events could maximize the efficiency of their calls. Sex pheromones are costly to produce (Harari et al. 2011), and female *M. sexta's* continued calling in an extremely high ozone-enriched environment demonstrates a lack of behavioral plasticity to rapidly changing atmospheric conditions. Furthermore, this finding contributes to earlier work suggesting that *M. sexta* cannot detect ozone, and would thus have one avenue of behavioral response to polluted atmospheres--evading pollution by moving out of high-pollution areas--closed to them.

Ozone exposure has very limited impact on insect antennal response

We predicted that ozone would damage antennal olfactory receptors, and as a result, moth antennae exposed to ozone would be less sensitive to odors. In agreement with this prediction, we find that antennae from male moths that had been exposed to ozone on three consecutive days had a lower sensitivity to the odor linalool at a very low concentration (10^{-4}) relative to antennae from male moths exposed only to clean air for three days. This decrease in sensitivity was not significant for other concentrations of linalool, but response to linalool was consistently lower for ozone-exposed antenna (Fig.3A). However, this increased detection threshold in ozone-exposed antennae did not manifest in response to the odors cis-3-hexenyl benzoate or methyl salicylate (Fig.3B). Methyl salicylate and linalool both elicited large responses from the insect antenna, demonstrating a high-abundance of receptors that can bind with both of these compounds. However, only linalool receptors seemed to have been damaged by ozone, so it appears that abundance does not predict whether the OR will be damaged by ozone. The effects of ozone on receptors may be caused by differences in the structure and permissivity of ORs. While linalool and methyl salicylate can both bind with many ORs on the antenna (Fraser 2003), linalool is an oxygenated alcohol monoterpene, while methyl salicylate is an ester aromatic.

Different classes of chemical compounds can activate different olfactory receptor types, and previous work examining the response of 35 male *M. sexta* ORs to a range of odorants found no ORs that were activated by both linalool and methyl salicylate, even though methyl salicylate activated 20 out of 35 ORs and linalool activated eight (Shields and Hildebrand 2001). If ozone damage is receptor-specific, it is possible that ozone could damage olfactory receptors for pheromone reception, but given the limited damage found for the three odors we tested, a more precise method, such as single sensillum recordings, may be necessary to detect ozone damage to olfactory sensitivity to pheromone compounds.

Broader Impacts

Insect species are facing worldwide declines (Potts et al. 2010; Kluser et al. 2007; Hallman et al. 2017), and moth species in particular have had dramatic documented declines in recent decades (Fox et al. 2013; Conrad et al. 2006). Currently, many anthropogenic culprits have been identified as potential drivers of these reported declines, and anthropogenically increased tropospheric oxidants should be considered as a contributing stressor to such declines, as decreases in efficiency or ability to locate mates via sex pheromones could have dramatic effects on species abundance. This study finds that ozone can alter *M. sexta* pheromone components, and cause pheromone plumes to be less attractive to mate-searching *M. sexta* males, opening the door to further studies examining the impacts of oxidants on pheromone plume discrimination by multiple moth species. Since ozone and other oxidants vary temporally and spatially, tropospheric pollutants have the potential to affect moths based on their location and density which dictate the likelihood of encountering a plume at a close distance, as well as based on the reactivity of the pheromone plume used to attract mates. Further work is needed across moth species to investigate whether the troubling degradation of pheromone plumes impacts moth's

ability to locate females in the field, and whether inefficiencies in mate location in polluted atmospheres could perniciously impact insect populations.

Citations

Allison, J. D., & Carde, R. T. (2016). *Pheromone communication in moths: evolution, behavior, and application*. Univ of California Press.

Ando, T., Inomata, S. I., & Yamamoto, M. (2004). Lepidopteran sex pheromones. In *The Chemistry of Pheromones and Other Semiochemicals I* (pp. 51-96). Springer, Berlin, Heidelberg.

Atema, J., & Engstrom, D. G. (1971). Sex pheromone in the lobster, Homarus americanus. *Nature*, *232*(5308), 261.

Atkinson, R., Carter, W. P., Plum, C. N., Winer, A. M., & Pitts Jr, J. N. (1984). Kinetics of the gas-phase reactions of NO3 radicals with a series of aromatics at 296±2 K. *International journal of chemical kinetics*, *16*(7), 887-898.

Austin, C. J., Guglielmo, C. G., & Moehring, A. J. (2014). A direct test of the effects of changing atmospheric pressure on the mating behavior of Drosophila melanogaster. *Evolutionary ecology*, *28*(3), 535-544

Baker, T. C., Cossé, A. A., Lee, S. G., Todd, J. L., Quero, C., & Vickers, N. J. (2004). A comparison of responses from olfactory receptor neurons of Heliothis subflexa and Heliothis virescens to components of their sex pheromone. *Journal of Comparative Physiology A*, *190*(2), 155-165.

Bocci, V. (2005). *How does ozone act? How and why can we avoid ozone toxicity?* (pp. 19-28). Springer Netherlands.

Carlsson, M. A., Galizia, C. G., & Hansson, B. S. (2002). Spatial representation of odours in the antennal lobe of the moth Spodoptera littoralis (Lepidoptera: Noctuidae). *Chemical senses*, *27*(3), 231-244.

Carlsson, M. A., & Hansson, B. S. (2006). 11 Detection and Coding of Flower Volatiles in Nectar-Foraging Insects. *Biology of floral scent*, 243.

Christensen, T. A., & Hildebrand, J. G. (1987). Male-specific, sex pheromone-selective projection neurons in the antennal lobes of the moth Manduca sexta. *Journal of comparative Physiology A*, *160*(5), 553-569.

Conrad K.F Warren, M. S., Fox, R., Parsons, M. S., & Woiwod, I. P. (2006). Rapid declines of common, widespread British moths provide evidence of an insect biodiversity crisis. *Biological conservation*, *132*(3), 279-291

Descoins, C., Silvain, J. F., Lalanne-Cassou, B., & Cheron, H. (1988). Monitoring of crop pests by sexual trapping of males in Guadeloupe and Guyana. *Agriculture, Ecosystems & Environment*, *21*(1-2), 53-56.

Date, E. M., Lemon, R. E., Weary, D. M., & Richter, A. (1991). Species identity by birdsong: discrete or additive information?. *Animal Behaviour*, *41*(1), 111-120.

Farré-Armengol, G., Peñuelas, J., Li, T., Yli-Pirilä, P., Filella, I., Llusia, J., & Blande, J. D. (2016). Ozone degrades floral scent and reduces pollinator attraction to flowers. *New Phytologist*, *209*(1), 152-160.

Fiore, A. M., Jacob, D. J., Bey, I., Yantosca, R. M., Field, B. D., Fusco, A. C., & Wilkinson, J. G. (2002). Background ozone over the United States in summer: Origin, trend, and

contribution to pollution episodes. *Journal of Geophysical Research: Atmospheres*, *107*(D15).
Fox, R. (2013). The decline of moths in Great Britain: a review of possible causes. *Insect Conservation and Diversity*, *6*(1), 5-19.
Fraser, A. M., Mechaber, W. L., & Hildebrand, J. G. (2003). Electroantennographic and behavioral responses of the sphinx moth Manduca sexta to host plant headspace volatiles. *Journal of chemical ecology*, *29*(8), 1813-1833.
Fuentes, J. D., T'ai, H. R., & Zenker, J. (2013). Ozone impedes the ability of a herbivore to find its host. *Environmental Research Letters*, *8*(1), 014048.
Goldstein, B. D., Lodi, C., Collinson, C., & Balchum, O. J. (1969). Ozone and lipid peroxidation. *Archives of Environmental Health: An International Journal*, *18*(4), 631-635.
Greenfield, M. D. (1981). Moth sex pheromones: an evolutionary perspective. *The Florida Entomologist*, *64*(1), 4-17.
Gomez-Diaz, C., & Benton, R. (2013). The joy of sex pheromones. *EMBO reports*, *14*(10), 874-883.
Hallmann CA, Sorg M, Jongejans E, Siepel H, Hofland N, Schwan H, et al. More than 75 percent decline over 27 years in total flying insect biomass in protected areas. Plos One. 2017;12(10)
Hansson, B. S., Christensen, T. A., & Hildebrand, J. G. (1991). Functionally distinct subdivisions of the macroglomerular complex in the antennal lobe of the male sphinx moth Manduca sexta. *Journal of comparative Neurology*, *312*(2), 264-278.
Hansson, B. S., Carlsson, M. A., & Kalinova, B. (2003). Olfactory activation patterns in the antennal lobe of the sphinx moth, Manduca sexta. *Journal of Comparative Physiology A*, *189*(4), 301-308.
Harari A. R., Zahavi, T., & Thiéry, D. (2011). Fitness cost of pheromone production in signaling female moths. *Evolution: International Journal of Organic Evolution*, *65*(6), 1572-1582.
Hauglustaine, D. A., & Brasseur, G. P. (2001). Evolution of tropospheric ozone under anthropogenic activities and associated radiative forcing of climate. *Journal of Geophysical Research: Atmospheres*, *106*(D23), 32337-32360.
Heath, R. L. (1980). Initial events in injury to plants by air pollutants. *Annual review of plant physiology*, *31*(1), 395-431.
Heath, R. L. (1994). Possible mechanisms for the inhibition of photosynthesis by ozone. *Photosynthesis Research*, *39*(3), 439-451.
Heinbockel, T., Kloppenburg, P., & Hildebrand, J. G. (1998). Pheromone-evoked potentials and oscillations in the antennal lobes of the sphinx moth Manduca sexta. *Journal of Comparative Physiology A*, *182*(6), 703-714.
Hillier, N. K., & Baker, T. C. (2016). Pheromones of Heliothine moths. In *Pheromone Communication in Moths-Evolution, Behavior, and Application* (pp. 301-333). University of California Press.
Kaae, R. S., H. H. Shorey, S. U. McFarland, and L. K. Gaston. (1973). Sex pheromones of Lepidoptera. XXXVII. Role of sex pheromones and other factors in reproductive isolation among ten species of Noctuidae. Annals of the Entomological Society of America 66:444–448.
Kaissling, K. E., Hildebrand, J. G., & Tumlinson, J. H. (1989). Pheromone receptor cells in the male moth Manduca sexta. *Archives of Insect Biochemistry and Physiology*, *10*(4), 273-279.
Karlson, P., & Lüscher, M. (1959). 'Pheromones': a new term for a class of biologically active substances. *Nature*, *183*(4653), 55.

Karlson, P., & Butenandt, A. (1959). Pheromones (ectohormones) in insects. *Annual review of entomology*, *4*(1), 39-58.
Kent, K. S., Hoskins, S. G., & Hildebrand, J. G. (1987). A novel serotonin-immunoreactive neuron in the antennal lobe of the sphinx moth Manduca sexta persists throughout postembryonic life. *Journal of neurobiology*, *18*(5), 451-465.
Klumpp, A., Bauer, E., Huttenlocher, F., Veit, U., & Gonschorrek, J. (2000). Effects of realistic ozone concentrations on the sex pheromone of the green oak tortrix (Tortrix viridana L.). *Mitteilungen der Deutschen Gesellschaft für allgemeine und angewandte Entomologie*, *12*(1-6), 67-70.
Kluser, S., & Peduzzi, P. (2007). Global pollinator decline: a literature review.
Landolt, P.J., C. l. smithhisler, r. s. Zack, and l. Camelo. (2006). Attraction of Heliothis belladonna (henry and edwards) to the sex pheromone of the corn earworm moth, Helicoverpa zea (Boddie) (lepidoptera: noctuidae). *Journal of the Kansas Entomological Society* 79:303–308.
Li, T., Blande, J. D., & Holopainen, J. K. (2016). Atmospheric transformation of plant volatiles disrupts host plant finding. *Scientific Reports*, 6.
Löfstedt, C., Herrebout, W. M., & Menken, S. B. (1991). Sex pheromones and their potential role in the evolution of reproductive isolation in small ermine moths (Yponomeutidae). *Chemoecology*, *2*(1), 20-28.
Matsumoto, S. G., & Hildebrand, J. G. (1981). Olfactory interneurons in the moth Manduca sexta: response characteristics and morphology of central neurons in the antennal lobes. *Proceedings of the Royal Society of London. Series B. Biological Sciences*, *213*(1192), 249-277.
Mondor, E. B., Tremblay, M. N., Awmack, C. S., & Lindroth, R. L. (2004). Divergent pheromone-mediated insect behaviour under global atmospheric change. *Global Change Biology*, *10*(10), 1820-1824.
Monks, P. S. (2005). Gas-phase radical chemistry in the troposphere. *Chemical Society Reviews*, *34*(5), 376-395.
Miura, Nami, et al. "A male-specific odorant receptor conserved through the evolution of sex pheromones in Ostrinia moth species." *International journal of biological sciences* 5.4 (2009): 319.
Naik, V., Voulgarakis, A., Fiore, A. M., Horowitz, L. W., Lamarque, J. F., Lin, M., ... & Cionni, I. (2013). Preindustrial to present-day changes in tropospheric hydroxyl radical and methane lifetime from the Atmospheric Chemistry and Climate Model Intercomparison Project (ACCMIP). *Atmospheric Chemistry and Physics*, *13*(10), 5277-5298.
Nesbitt, B. F., Beevor, P. S., Cole, R. A., Lester, R., & Poppi, R. G. (1973). Sex pheromones of two noctuid moths. *Nature New Biology*, *244*(137), 208.
Pellegrino, A. C., Peñaflor, M. F. G. V., Nardi, C., Bezner-Kerr, W., Guglielmo, C. G., Bento, J. M. S., & McNeil, J. N. (2013). Weather forecasting by insects: modified sexual behaviour in response to atmospheric pressure changes. *PLoS One*, *8*(10), e75004.
Potts, S. G., Biesmeijer, J. C., Kremen, C., Neumann, P., Schweiger, O., & Kunin, W. E. (2010). Global pollinator declines: trends, impacts and drivers. *Trends in ecology & evolution*, *25*(6), 345-353.
Raina, A. K., J.A. Klun, J. D. Lopez, and B. A. Leonhardt. (1986). Female sex pheromone of Heliothis phloxiphaga (Lepidoptera: Noctuidae): chemical identification, male behavioral response in the flight tunnel, and field tests. Environmental Entomology 15: 931–935.
Rasmussen, L. E. L., Lee, T. D., Zhang, A., Roelofs, W. L., & Daves Jr, G. D. (1997). Purification, identification, concentration and bioactivity of (Z)-7-dodecen-1-yl acetate:

sex pheromone of the female Asian elephant, Elephas maximus. *Chemical Senses*, *22*(4), 417-437.
Roelofs, W. L., & Comeau, A. (1969). Sex pheromone specificity: taxonomic and evolutionary aspects in Lepidoptera. *Science*, *165*(3891), 398-400.
Roelofs, W.L., A. S. Hill, R. T. Cardé, and T. C. Baker. (1974). Two sex pheromone components of the tobacco budworm moth, Heliothis virescens. Life Sciences 14:1555–1562.
Roehm, J. N., Hadley, J. G., & Menzel, D. B. (1971). Oxidation of unsaturated fatty acids by ozone and nitrogen dioxide. *Archives of Environmental Health: An International Journal*, *23*(2), 142-148.
Sakurai, T., Nakagawa, T., Mitsuno, H., Mori, H., Endo, Y., Tanoue, S., ... & Nishioka, T. (2004). Identification and functional characterization of a sex pheromone receptor in the silkmoth Bombyx mori. *Proceedings of the National Academy of Sciences*, *101*(47), 16653-16658..
Sakurai, T., Mitsuno, H., Haupt, S. S., Uchino, K., Yokohari, F., Nishioka, T., ... & Kanzaki, R. (2011). A single sex pheromone receptor determines chemical response specificity of sexual behavior in the silkmoth Bombyx mori. *PLoS genetics*, *7*(6), e1002115.
Schaich, K. M., & Pryor, W. A. (1980). Free radical initiation in proteins and amino acids by ionizing and ultraviolet radiations and lipid oxidation—Part III: Free radical transfer from oxidizing lipids. *Critical Reviews in Food Science & Nutrition*, *13*(3), 189-244.
Shields, V. D. C., & Hildebrand, J. G. (2001). Responses of a population of antennal olfactory receptor cells in the female moth Manduca sexta to plant-associated volatile organic compounds. *Journal of Comparative Physiology A*, *186*(12), 1135-1151.
Stanger-Hall, K. F., & Lloyd, J. E. (2015). Flash signal evolution in Photinus fireflies: character displacement and signal exploitation in a visual communication system. *Evolution*, *69*(3), 666-682.
Stelinski, L., Holdcraft, R., & Rodriguez-Saona, C. (2014). Female moth calling and flight behavior are altered hours following pheromone autodetection: possible implications for practical management with mating disruption. *Insects*, *5*(2), 459-473.)
Symonds, M. R., & Elgar, M. A. (2008). The evolution of pheromone diversity. Trends in ecology & evolution, 23(4), 220-228.
Teal, P.E.A., J. H. Tumlinson, J. R. McLaughlin, R. Heath, and R. A. Rush. 1984. (Z)-11-Hexadecen-1-ol: a behavioral modifying chemical present in the pheromone gland of female Heliothis zea (Lepidoptera: Noctuidae). Canadian Entomologist 116:777–779
Tumlinson, J. H., Brennan, M. M., Doolittle, R. E., Mitchell, E. R., Brabham, A., Mazomenos, B. E., ... & Jackson, D. M. (1989). Identification of a pheromone blend attractive to Manduca sexta (L.) males in a wind tunnel. *Archives of Insect Biochemistry and Physiology*, *10*(4), 255-271.
Tilton, B. E. (1989). Health effects of tropospheric ozone. *Environmental Science & Technology*, *23*(3), 257-263.
Vickers, n. J. (2002). Defining a synthetic pheromone blend attractive to male Heliothis subflexa under wind tunnel conditions. Journal of Chemical Ecology 28:1255–1267
Vickers, N. J. (2017). Animal Communication: When I'm Calling You, Will You Answer Too?. *Current Biology*, *27*(14), R713-R715.
Victorin, K. (1992). Review of the genotoxicity of ozone. *Mutation Research/Reviews in Genetic Toxicology*, *277*(3), 221-238.
Vingarzan, R. (2004). A review of surface ozone background levels and trends. *Atmospheric Environment*, *38*(21), 3431-3442.

Wanner Kevin W., Andrew S. Nichols, Jean E. Allen, Peggy L. Bunger, Stephen F. Garczynski, Charles E. Linn Jr, Hugh M. Robertson, and Charles W. Luetje. "Sex pheromone receptor specificity in the European corn borer moth, Ostrinia nubilalis." *PloS one* 5, no. 1 (2010): e8685.

Wilson, R. I., Turner, G. C., & Laurent, G. (2004). Transformation of olfactory representations in the Drosophila antennal lobe. *Science*, *303*(5656), 366-370.

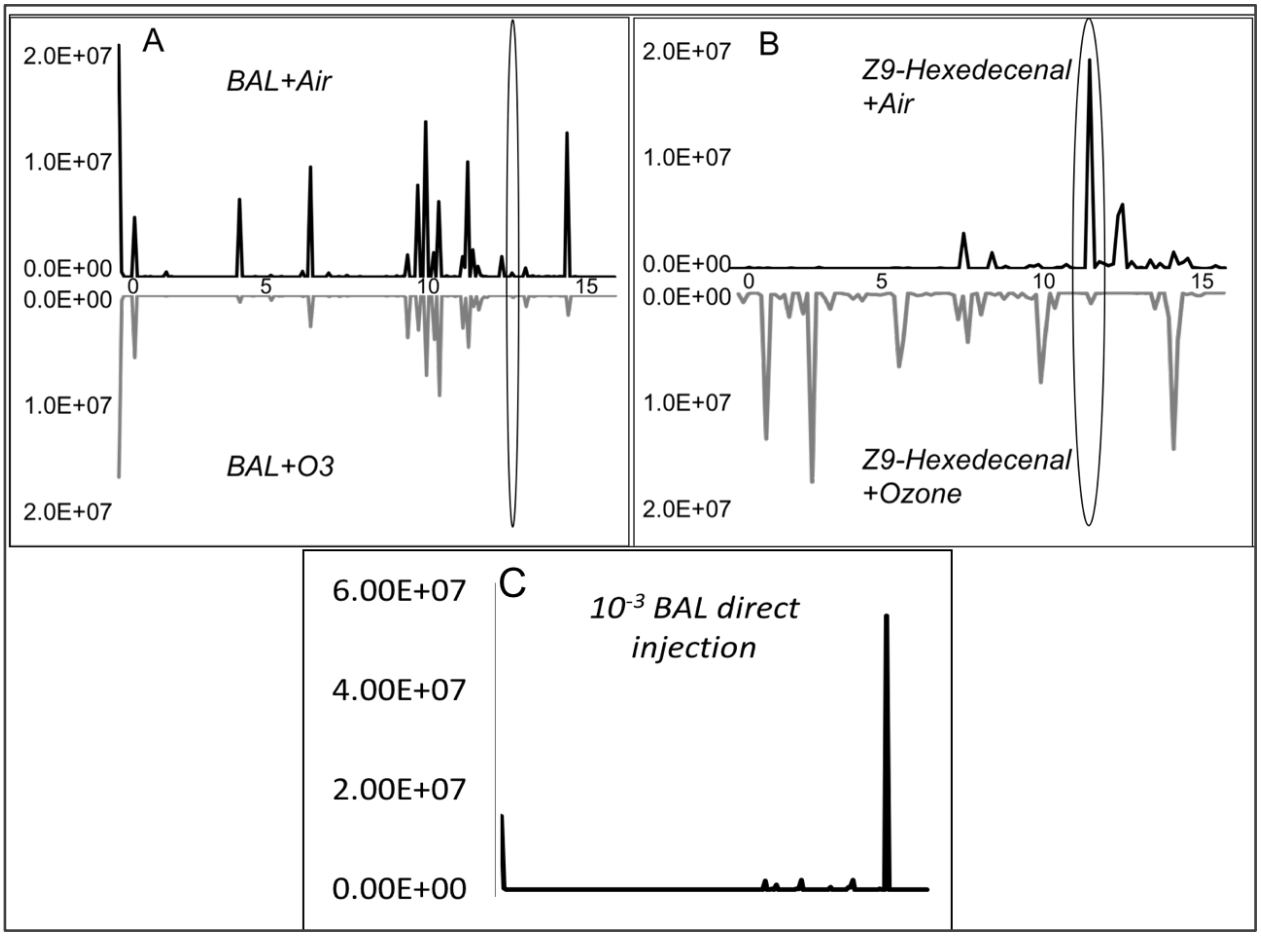
Wilson, R. I., & Laurent, G. (2005). Role of GABAergic inhibition in shaping odor-evoked spatiotemporal patterns in the Drosophila antennal lobe. *Journal of Neuroscience*, *25*(40), 9069-9079.

Young PJ, et al. (2013), Pre-industrial to end 21st century projections of tropospheric ozone from the Atmospheric Chemistry and Climate Model Intercomparison Project (ACCMIP). *Atmos. Chem. Phys* 13: 2063–2090.

Zagvazdina, N. Y., Paris, T. M., Udell, B. J., Stanislauskas, M., McNeill, S., Allan, S. A., & Mankin, R. W. (2015). Effects of atmospheric pressure trends on calling, mate-seeking, and phototaxis of Diaphorina citri (Hemiptera: Liviidae). *Annals of the Entomological Society of America*, *108*(5), 762-770.

Zhang, J.-P., C. salcedo, Y.-l. fang, r.-l. Zhang, and Z.-n. Zhang. (2012). An overlooked component: (Z)-9-tetradecenal as a sex pheromone in Helicoverpa armigera. Journal of Insect Physiology 58:1209–1216.

Supplementary Material



S1. Traces of single compounds exposed to ozone and air collected on SuperQ for 12 hours. **A.** 100µl of 10^{-2} Bombykal mixed with air (black trace) and ozone (grey trace) and collected on SuperQ for 12 hours. Only a small amount of bombykal was collected in air or ozone-mixed plumes (grey circle). **B** 100µl of 10^{-2} (Z)-9-hexadecenal mixed with air (black trace) and ozone (grey trace) and collected on SuperQ for 12 hours. While (Z9)-hexadecenal was still abundant in plumes of (Z)-9-hexadecenal mixed with air, only a small amount of (Z9)-hexadecenal was collected in ozone-mixed plumes (grey circle). **C**. 1µl of 10^{-3} bombykal in hexane injected directly into the GC-MS shows minimal but visible amounts of breakdown products.

Chapter #3

Ozone-altered floral plumes are less attractive to a specialist but not a generalist herbivore, but the specialist herbivore can learn ozone-altered plumes

Summary

Foraging insects use floral plumes as 'scent pathways' that they can follow through a landscape to their preferred host plants. However, elevated levels of tropospheric ozone could react with these floral plumes, making them less attractive to foraging insects. Specialist and generalist insects, however, typically use different components of a plume and may thus be differentially impacted by elevated ozone levels. Specialist insects may rely on an innate attraction to uniquely identifying 'token' compounds, while generalists can find an array of common floral volatile compounds attractive and hone their preferences based on experience. Thus, unlike generalists, specialists' responses to ozone-exposed plumes are likely to depend on the ozone-reactivity of just a few key compounds. Here, we determine that a specialist finds two key compounds attractive and that the specialist's response to those key compounds is determined by their ozone reactivity in ozone-enriched environments. However, we further determine that the specialist but not the generalist finds ozone-altered plumes of a shared host plant to be less attractive, despite the presence of an unreactive key compound. This loss of attraction may be driven by avoidance of the breakdown products of ozone with other plume components. However, after feeding on the host-plant in an ozone-enriched environment, the specialist increased its attraction to the ozone-mixed floral plume, demonstrating that a specialist is able to learn an initially unattractive floral scent. This work contributes to the growing body of literature investigating the impacts of ozone on insect foraging via olfaction and demonstrates that individual insects' response to ozone-exposed plumes may not be easily ascribed to foraging type.

INTRODUCTION

Host location by olfactory cues

Herbivorous insects use both visual and olfactory cues to locate their host plants (Prokopy and Owens 1983; Reeves 2011; Kunze and Gumbert 2001). While visual cues may be the primary mode of host-selection for some herbivores (e.g. Stenberg and Ericson 2007), olfactory cues also play an important role in host-plant location and are particularly critical at long distances beyond the range of visual cues. Olfactory cues are comprised of blends of volatile organic compounds (VOCs) that are emitted from both floral and leaf tissue (Dudareva and Pichersky 2006). Blends of VOCs move downwind and create an array of scent filaments interspersed with pockets of clean air. Foraging insects can follow these scent filaments through the landscape to their host plants. While floral volatiles function primarily to attract pollinators, eavesdropping herbivores can also use floral plumes to detect and locate their host plants (Irwin et al. 2004; Schiestl et al. 2011; Theis 2006; Theis and Adler 2005; Knauer and Schiestl 2017). A foraging insect can use the composition of the floral plume to identify its host plants, recognizing the plume either by the presence or absence of specific compounds (Hansson et al. 1999; Burger et al. 2010, Milet-Pinhero et al. 2012), by blends of different compounds (Riffell et al. 2009; Bruce et al. 2005; Bruce and Picketts 2011), or by specific ratios of some compounds in a blend (Bruce et al. 2005). Once an insect has identified a plume as belonging to a viable host plant, it can use this plume to navigate towards that host plant through a landscape (Visser 1988; Bell et al. 1990).

Plumes may be heavily perturbed by air pollution

The olfactory cues that insects use to locate their host plants, however, may be degraded by atmospheric pollution. Atmospheric pollutants have increased dramatically as a result of heightened hydrocarbon combustion from power plants and automobiles in the wake of the industrial revolution. Primary pollutants that result from this combustion further react in the atmosphere to form secondary pollutants, including the highly reactive oxidant ozone (Davis et al. 1997; Ryerson et al. 2011). For North America, current levels of ozone are approximately 30% higher than pre-industrial times (Young et al. 2013), with some predictions that ozone will increase by as much as 120% by 2050 for northern mid-latitudes in July (Brausser et al. 2001). Currently, ozone levels can reach peaks of 120 ppbv in North America during summertime ozone events (Vingarzan 2004). As a strong oxidant, ozone can break apart some of the carbon-carbon double bonds present in many VOCs, potentially interfering with insect foraging both by decreasing the distance that reactive VOCs can travel through the atmosphere and by altering the composition of blends of VOCs. In the troposphere, ozone reacts with a specific VOC based on both the concentration of the ozone and the reactivity of a given individual compound with ozone (Jacob 1999; Atkinson and Arey 2003). To date, the majority of VOCs tested are reactive with ozone, but some exceptions exist (Atkinson and Arey 2003; Baker et al 2001). Because the plume of a single plant contains many different VOCs, exposing a plume to ozone can simultaneously reduce the abundance of individual compounds and change the relative abundance of plume constituents, depending on their reactivity with ozone (Lusebrink et al. 2015; McFrederick et al. 2008; Farre-Armengol et al. 2016). Moreover, when ozone reacts with a VOC, it generates reaction products. Such reaction products may include long-lived secondary VOCs, but may also include compounds that are very dissimilar in structure and function from parent VOCs: two common breakdown products are formaldehyde and acetone (McFrederick et al. 2009; Lee at al 2006). Ozone-induced changes to the plume composition occur as the plume

moves downwind from the host plant, creating a gradient of increasingly altered plumes with increasing distance from the host plant (McFrederick et al. 2009; Farre-Armengol et al. 2016). Thus, at distances far from their host plants, foraging insects may encounter plumes so altered by ozone that the plumes are significantly less attractive or even unrecognizable. Emerging lab studies on a taxonomically diverse group of insects, including a striped cucumber beetle (Fuentes et al. 2013), a bumblebee (Farre-Armengol et al. 2016), a diamondback moth larva (Li et al. 2016), and an adult sphinx moth (Cook et al. in prep 2019), have found that ozone-altered floral plumes are less attractive to foraging insects.

Floral cue use and availability post-ozonation could lead to different foraging outcomes

Because ozone reacts with most but not all VOCs in a plume (Atkinson and Arey 2003), the response of an insect to an ozone-altered plume will depend on which specific VOCs in the plume that insect uses for host location. Thus, insect response to an ozone-altered plume will be very specific to both insect and plant species (with the likelihood that ozone reaction with plumes is unlikely to make them more attractive). More broadly, however, insects with different foraging strategies may utilize olfactory cues differently, potentially leading to divergent foraging success in ozone enriched environments. Insects may be divided into two classes based on their dietary needs, with specialist insects feeding on just a few taxonomically related species (Bernays and Champman) and generalists insects feeding on a wide array of host plants from different families (Bruce and Picketts 2011). Based on these different dietary restrictions, generalists and specialists may employ different olfactory cue-use strategies. Specialist insects may express an innate attraction to VOCs that are unique identifiers or ‘token compounds’ (G.S. Fraenkel 1959) of their few host plants. In contrast to specialists, generalist insects may employ a two-step cue-use strategy, wherein they exhibit an innate attraction to many common VOCs

(Lance 1983) but then develop a preference for specific olfactory cues based on their feeding experience (Bruce and Picketts 2011; Bruce et al. 2005).

Evidence for divergent specialist and generalist foraging strategies

A growing number of studies support the idea that specialist insects are far more restricted than generalists, not only in the breadth of their diet but also in the number of olfactory cues that they can associate with host flowers. Specialists' use of 'token compounds' has been best studied in oligolectic bees, including bees that collect oil from oil-producing plants (Schaffler et al. 2015) and two oligolectic bees, *Chelostoma rapunculi* and *Hoplitis adunca,* which both demonstrate a strong antennal and behavioral response to unusual VOCs currently identified only in the plumes of their host plants (Milet-Pinheiro et al. 2012; Burger et al. 2012; Brandt et al. 2017). A further comparative study on the antennal responses of three generalist versus three specialist bees, including *C. rapunculi,* found that while specialist and generalist bees could detect many of the same common compounds, the specialist insects had additional olfactory receptors that were fine-tuned to unusual compounds of their host plants (Brandt et al. 2017). Such fine-tuned olfactory receptors for token compounds have also been identified in the specialist cabbage seed weevil (Blight et al. 1989) and cabbage aphid (Nottingham et al. 1991).

Specialists' innate attraction to token compounds could enable them to locate host plants without the need for costly exploration and learning. While there is currently a dearth of studies on olfaction-learning in specialist insects, recent studies on oligolectic bees report mostly limited learning abilities for these specialist insects (Milet-Pineheiro et al. 2012; Burger et al. 2010; Milet-Pinheiro et al. 2013; Dotterl et al. 2005; Dobson et al. 2000), although specialist parasitoids have shown strong abilities to learn olfactory cues (Turlings et al. 1993; Stiedle et al. 1997; Potting et al. 1999). Further studies on the oligolectic bees *C. rapunculi* and *H. adunca* as

well as another oligolectic bee, *Andrena vaga,* found that these bees demonstrate only limited learning abilities, either increasing their reliance on visual cues after foraging, losing their attraction to some key compounds, or expanding their olfactory repertoire after feeding to include some other compounds in their host-plant's bouquet (Milet-Pineheiro et al. 2012; Burger et al. 2010; Milet-Pinheiro et al. 2013; Dotterl et al. 2005; Dobson et al. 2000). Specialists' relatively limited learning abilities are in strong contrast to generalist insects' learning abilities, which have been demonstrated in a large and taxonomically diverse number of generalist insects. The ability to learn complex olfactory cues in myriad ways is perhaps best showcased in the large number of studies on olfactory learning in generalist bees, including the honeybee *Apis mellifera* (Hammer and Menzel 1995) and at least three bumblebee (*Bombus*) species (Sommerlandt et al. 2014; Riveros & Gronenberg 2009). However, olfactory learning has also been demonstrated in locusts (Simoes et al. 2011), bark beetles (Costa, & Reeve 2011), and several generalist phytophagous insects (Papaj and Prokopy 1989).

Predictions for specialists in ozone enriched-environments

Given differences in the salient compounds in a plume for generalist and specialist insects and potential differences in their olfactory learning abilities, specialists and generalists foraging in ozone-enriched environments are likely to have their perceptions of plumes altered by ozone in different ways. This potentially leads to divergent outcomes in foraging success, even if specialist and generalist insects use the same ozone-altered plume to locate a shared host plant. The generalist's use of many VOCs make it likely that some, but not all, of the compounds relevant to a generalist are reactive with ozone, potentially resulting in a somewhat buffered decrease in attraction to ozonated plumes, rather than a complete loss of attraction. This type of decrease in attraction rather than total attraction loss has been seen for the generalist *Bombus*

terrestris responding to ozone-altered plumes of *Brassica nigra* (Farre-Armengol et al. 2016). Such a gradational response to an ozonated plume is in contrast to the likely binary outcome for specialists foraging in polluted atmospheres. For specialists, the ozone-reactivity of the token compounds they rely on could result in either unsullied attraction or complete loss of attraction to a plume exposed to ozone. In the case where token compounds are reactive with ozone, we would expect a specialist to lose its attraction to the ozone-exposed plume. In the case where token compounds are unreactive with ozone, a specialist forager may not be affected by the presence of ozone at all. In the case where one or more token compound is reactive with ozone, but at least one token compound remains unreacted, we would anticipate that the specialist would maintain its attraction to the ozonated plume, even in the face of a loss of other token compounds.

Study system and research questions

Assessing the response of generalist and specialist insect foragers to ozone-altered plumes of a host plant before and after experience with the ozone-altered plume can provide insights into how ozone could impact foraging for insects employing two different foraging strategies of cue-use and learning. Here, we compare the response of a specialist forager, the striped cucumber beetle *A. vittatum* (henceforward, the specialist), and a generalist forager, the spotted cucumber beetle *D. undecimpunctata* (henceforward, the generalist), to ozone-altered plumes of a shared host plant before and after feeding experience with the ozonated-plume. Previous studies have investigated olfactory cue use in both beetles (Lampman et al. 1987; Metcalf and Lampman 1989; Lampman and Metcalf 1990; Lewis et al. 1990; Metcalf and Lampman 1991; Metcalf et al. 1995) and have identified two candidate key compounds for *A. vittatum:* indole (Lewis et al. 1990; Adler et al. 2009) and 1,2,4-Trimethoxybenzene (Adler et al. 2009). According to a study

on three zucchini cultivars of *C. pepo*, Indole is produced exclusively in the petals, while 1,2,4-trimethoxybenzene is primarily produced in the floral nectar (Granero et al 2005). Both of these compounds in isolation can attract adult *A. vittatum* in the field, but one compound is highly reactive with ozone (Indole: reaction rate with ozone $KO_3 = 4.9*10^{-17}$), while the other is largely unreactive (1,2,4-Trimethoxybenzene: estimated reaction rate $KO_3=1*10^{-30}$). Investigating the response of the specialist to these individual compounds before and after ozonation, along with its response to the ozonated plume containing both compounds, will elucidate how ozone-reaction of some, but not all, key compounds impact specialist foraging response. Furthermore, this response can be compared to that of a generalist insect, to determine if initial or experienced responses to ozone-altered plumes differ based on insect foraging strategy. To ascertain the impacts of ozone on the attraction of both a specialist and generalist insect to a shared host plant, we propose the following four hypotheses:

HYPOTHESES

(1) Plumes of *Cucurbita pepo* flowers will be altered by exposure to 120 ppbv ozone, and the candidate key-compounds indole but not 1,2,4-trimethoxybenzene (124TMB) will be reduced via reaction with ozone both in the plume and individually.

(2) The specialist will find both indole and 124TMB attractive, however it will lose attraction to the reactive compound indole but not to the unreactive compound 124TMB when they are mixed with ozone. The generalist will find neither single compound attractive.

(3) The specialist will maintain its attraction to the *C. pepo* plume mixed with ozone following its attraction to the single unreactive compound 124TMB in ozone. The generalist will find the ozonated plumes less attractive than the pure plume but more attractive than air.

(4) Secondary products resulting from reactions between ozone and VOCs will deter both specialists and generalists. Specialists and generalists choosing between a two-compound mixture containing an unreactive compound that is attractive (124TMB) and an ozone-reactive compound that is unattractive (linalool) will prefer the two-compound blend in air vs. in ozone. **(5)** If ozone disrupts either beetle's attraction to the *C. pepo* plume, then generalists but not specialists will re-establish attraction via associative learning.

METHODS

Study organisms

Cucurbita pepo var crookneck or yellow squash, is a monecious summer squash. *Acalymma vittatum* is a specialist feeder of plants in the genus *Cucurbita* and a major pest on *Cucurbita* crops in north America (Brewer et al. 1987; Ellers-Kirk et al. 2006). Adult *A. vittatum* emerge in late spring, forage on the leaves, fruit, and flowers, including pollen and nectar, of *Cucurbita* (Andersen and Metcalf 1986, 1987; Metcalf and Metcalf 1992). *Acalymma vittatum* larvae feed on roots and overwinter near their host plants (Ellers-Kirk et al. 2000). *Diabrotica undecimpunctata howardi Barber* is another major agricultural pest in North America, where it feeds not only on cucurbits as its name, the spotted cucumber beetle, would imply, but also on other important crops, most notably corn (Brust and House 1990). Like *A. vittatum,* adult beetles feed on plant matter and will consume leaves, petals, pollen, and nectar of squash and other plants As larvae, *D. undecimpunctata* will feed on its host plant's roots (Krysan 1986).

Floral plumes of Cucurbita blossoms attract both *A. vittatum* (Andersen and Metcalf 1986, Lewis et al. 1990, Granero et al. 2004; Ferrari et al. 2006; Andrews et al. 2007; Fuentes et

al. 2013) and *Diabrotica undecimpunctata howardi Barber* (Jackson 2005), as found in studies where squash plants are put in a field, and adult *A. vittatum* and *D. undecimpunctata* locate them. Furthermore, existing work on behavior and GC-EAD have identified potential individual compounds that serve as attractants to both beetles (Lampman et al. 1987; Metcalf and Lampman 1989; Lampman and Metcalf 1990; Lewis et al. 1990; Metcalf and Lampman 1991; Metcalf et al. 1995).

Assessing the effects of ozone on Cucurbita pepo *floral plumes*

The floral plume of four *C. pepo* flowers was split into two flows, and each flow was then exposed to either 120 ppbv ozone or air to determine if ozone alters the *C. pepo* plume. Flowers of *C. pepo* used for odor collection were reared in a greenhouse at Max Planck Institute for Chemical Ecology in Jena, Germany. Plants were reared with a 16hr daylight schedule. To collect floral plumes from *C. pepo*, four flowers of *C. pepo* still attached to their host plant were put in a 1l glass odor-collection cylinder, with a teflon base which consisted of two halves of a circle with a cutout in the center, so that when the two halves were in place, the stems of the *C. pepo* flowers passed through the base, allowing the plant to remain outside of the chamber while the flowers were contained within the chamber. Odor collection occurred between 7-10am, when flowers were newly opened. Air that had been passed through a charcoal scrubber for cleaning entered the cylinder at a rate of 1.5l/min, and was pulled from the cylinder with a pump at a rate of 1l/min. The floral headspace was then split into two 0.5l/min flows and directed into two 2l airtight glass mixing bottles. In one mixing bottle, an additional 0.5l/min of ~120 ppbv ozone was added, while in the other bottle, 0.5l/min of scrubbed air was added. As floral plumes which had mixed with either 120 ppbv ozone or with scrubbed air left their respective mixing bottles, they passed through a small box containing 5 PDMS tubes, which collected the floral scent for

20 minutes. After the 20 minute floral headspace collection, a standard of 1µl of 10^{-6} bromohexane standard was added to each PDMS tube, and the PDMS tubes were either placed immediately in a -20°C freezer for later analysis, or analyzed immediately in a GC-MS. PDMS tubes were analyzed using a thermo desorption unit (TDU, Gerstel, Germany) coupled to a temperature-programmable vaporizing unit (CIS 4, Gerstel, Germany), which was linked to an Agilent 7890A gas chromatograph (Agilent Technologies, CA) running in splitless mode and being connected to an Agilent 5975C mass spectrometer (electron impact mode, 70eV, ion source: 230◦C, quadrupole: 150°C,mass scan range: 33–350u). We used a nonpolar column (HP-5 MS UI, 30m length, 0.25mm ID, 0.25µm film thickness, J and W Scientific, USA) under constant helium flow of 1.1ml/min. The TDU temperature raised from 30°C to 200°C at a rate of 100°C/min and held for 5min. Volatized compounds were trapped within the CIS 4 cooled injection system at -50°C and subsequently injected into the GC. The GC oven was programmed to hold 40°C for 3min, to increase the temperature at 5°C/min to 200°C, then to increase temperature at 20°C/min to 260°C, which was kept for 15min. Unprocessed data files were then exported and analyzed using the software package XCMS (Smith et al., 2006) implemented in R (R Core Team, 2014). Peak area values were log transformed to ensure normality and finally compared by a principal component analysis.

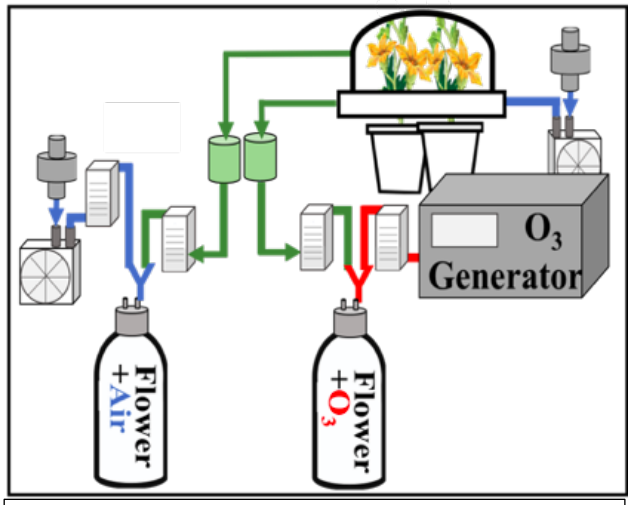


Diagram 1. Generation of ozonated vs. air-mixed floral plumes of *C. pepo* for chemical analysis

Plant and Insect acquisition and treatment

Both the specialist and the generalist beetles used in this study were collected either at Blandy Experimental Farm community garden in Boyce VA, or at WaterPenny Farm in Sperryville, VA. Once collected, the beetles were kept for at least one day (24hrs) in a mesh cage with a *C. pepo* plant so that all insects shared the same most recent feeding experience. After a minimum of one day in the mesh cage, beetles were starved for at least 16 hours in individual plastic vials before being used in choice assays assessing their attraction to individual plume components mixed with air or ozone, and the whole *C.pepo* plume mixed with air or with ozone.

Plants of *C. pepo* used in insect behavior assays were reared at Blandy Experimental Farm, Boyce, VA, and kept in a greenhouse during their development. *Cucurbita pepo* plants had a minimum of 16 hrs daylight supplemented with artificial light throughout their growth.

Choice tests

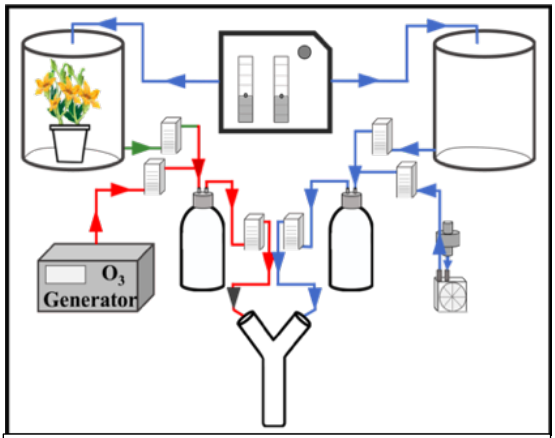


Diagram 2. Ozone-exposed *C. pepo* plumes vs. air used in behavior assays

Behavior tests were conducted over the course of two summers: from June-October of 2017 and 2018. Individual beetles that had starved for 16 hours in plastic vials were allowed to enter a y-tube to choose between two presented flows. Once they entered the y-tube, the beetles were given three minutes to make a choice between the two presented odors (or one odor and air) by entering one arm of the y-tube. If an insect made no choice after three minutes in the y-tube, it was recorded as "no choice." Insects were only used for one behavior assay before being removed from the study. Behavior assays in 2018 were filmed with a Sony handycam camera .

All choice assays were conducted in a lighted fumehood, with the base of the y-tube pointing towards the researcher outside the fume-hood.

A virtual coin flip was used to determine which arm of the y-tube emitted an odor vs. air or ozone-altered odor. The side of the y-tube at which the scent was emitted was switched after every 3-5 tested beetles, along with the upper vs. lower surfaces of the y-tube. After each beetle, the y-tube was cleaned using a kimwipe moistened with water, or moistened with acetone if the arms had switched odors. Every hour, the y-tube was cleaned with ethanol, acetone, and water. Only one odor was tested per day, and all glassware was cleaned nightly with ethanol, acetone, water, and ozone, and left overnight in an oven at 425^{o}F.

Ozone used in all behavior assays was generated using an ozone generator (Model 165, Thermo Scientific Inc., Pittsburg, PA), and ozone concentration was measured using an ozone analyzer (Model 202, 2B Technologies Inc., Boulder, CO).

Behavioral Assays

Response to plume components

To investigate how a specialist responds when one but not all of its key plume components are reactive with ozone, we began by verifying previous work identifying the single compounds indole and 124TMB as key attractants for the specialist. These behavior tests were conducted in the y-tube, where both the generalist and the specialist were given the choice between (1) indole and air, or (2) 124TMB and air. 35 beetles of each type were used in both of these assays. We next assessed the beetles' responses to these two compounds after mixing with ozone, to ensure that the reactive compound indole did indeed become less attractive and the unreactive compound 124TMB did not become less attractive after mixing with ozone. In the y-tube, beetles chose between the ozone-reactive indole mixed with air or mixed with ozone (3), or the

unreactive compound 124TMB mixed with either air or ozone (4). 40 beetles per assay were tested to assess the response of beetles to odors vs. odors in air. Odor plumes used for behavior assays were generated by applying 2µl of 10^{-2} of each compound (Sigma Aldrich, purity > 98%) dissolved in a solvent of 20% acetone 80% hexane to filter paper, and placing the filter paper in a 2l airtight bottle. Air enriched with 120 ppbv ozone flowed through the bottle at a rate of 0.5l/min, and 0.3l/min of the odor plume entered the y-tube. At the other arm of the y-tube, either scrubbed clean air or the same single odor mixed with scrubbed air entered the y-tube after having passed through an identical 2l bottle as the ozone-generated odor plume. Beetle preference for odors vs. air or odors in air vs. odors in ozone were tested using a chi-square.

Response to *C. pepo* plumes and ozonized *C. pepo* plumes

After ensuring that the specialist's response to single compounds matched our predictions, we next assessed the response of the specialist and generalist to the plume o*f C. pepo* exposed to air vs. exposed to 120 ppbv ozone. The plume of *C. pepo* mixed with air and *C. pepo* mixed with ozone were generated by placing a plant with at least four *C. pepo* flowers in an airtight glass chamber, with a steady flow of 0.5l/min through the chamber. The chamber was then connected to an airtight glass mixing bottle (2l) with two inlets: the flow from the chamber from the flower (~0.5l/min) and an input of 0.5l/min of either scrubbed air or 120 ppbv ozone. All flows entering the y-tube were set to 0.3l/min per arm. With the plumes thus generated, individual beetles were given the choice between either (5) the unaltered plume vs. air or (6) the ozone-altered plume vs. air. 40 beetles were tested for their response in each assay. 50 beetles of each type were used in the assays assessing beetle preference pure or ozonated plumes vs. air.

Response after experience with ozonized plumes and ability to learn a single compound

To determine if either the specialist or the generalist could learn the ozone-altered plume of *C. pepo*, we assigned beetles to ozone-enriched chambers where they could feed on *C. pepo* plants. Individual beetles were randomly assigned to one of six glass vial chambers with a screw-tight Teflon-lined lid. All six chambers contained a piece of leaf and a small squash flower. All six of these chambers had a steady flow of 120 ppbv ozone (~0.2l/min) entering through a puncture in the lid, and silicone wax was used to stop excess leakage. These chambers were kept in the fume-hood to remove excess ozone. Insects were kept in these chambers for at least 24 hours before being starved for 16 hours and used in behavior assays.

After their experience feeding on the ozone-altered plume, beetles were tested for their response to either the pure plume vs. air, or the ozone-altered plume vs. air, repeating the same choice assays (5) and (6): air vs. the plume of four *C. pepo* flowers mixed with air, and air vs the plume of four *C. pepo* flowers mixed with 120 ppbv ozone.

Beetle preference for floral odors vs. air or floral odors mixed with ozone vs. air were tested using a chi-square test.

We further investigated the beetle's ability to learn a single compound by testing the beetle's innate and trained response to the compound linalool, which is in some *C. pepo* plumes (Ferrari et al. 2006; Granero et al. 2005) but has not been documented as an attractant. We tested the beetle's response to linalool vs. scrubbed air in the y-tube, and then allowed the beetles to feed for at least 24 hours on 35% sugar water spiked with 10^{-8} linalool which was barely detectable to the human nose. After 24 hours, beetles that had been observed feeding on the sugar water were placed in individual plastic vials and starved for 16 hours before being tested for their response to linalool vs. air in the y-tube. Beetle's preference for linalool vs. air before and after training was assessed via chi-square tests comparing preference for linalool vs. air in pre and post-trained beetles.

Assessment of reaction products as deterrents for a single attractive compound

To better assess if secondary products that result from the reaction of ozone with primary floral compounds had an impact on beetle behavior in response to a plume, we included behavior tests using a sample reactive floral compound, linalool. Linalool is a component of some *Cucurbita* floral plumes (Ferrari et al. 2006; Granero et al. 2005; Shapiro et al. 2001), is a ubiquitous floral volatile (Knudsen et al. 1993), and is highly reactive with ozone.. Initially, we tested the beetles' responses to linalool vs. air to ensure that linalool by itself was not attractive. After finding that linalool alone was not attractive to either beetle, we further tested the beetles' responses to a mixture of the unreactive, attractive compound 124TMB, and the unattractive, reactive compound linalool mixed either in air or in ozone-enriched (120 ppbv) air. We expected that if linalool breakdown products were deterrent to the beetles, then they would prefer the 124TMB+linalool blend mixed with air rather than the blend mixed with ozone, which would react with linalool and generate secondary products. 35 beetles of each type were tested for their initial and post-training response to linalool, and preference for linalool +124TMB in air vs. ozone was tested with a chi-square test, as well as preference for 124TMB vs. 124TMB+Linalool.

Response to ozone vs. air and solvents vs. air

The potential deterrent effect of ozone itself was tested in another behavior assay, where both beetles were given a choice between either air scrubbed through charcoal, or scrubbed air enriched with 120 ppbv ozone. Beetles were also tested for their response to the 80% hexane, 20% acetone solvent used to dissolve single compounds vs. air to ensure that response to

solvents did not explain the behavioral responses we found to individual compounds. 35 beetles of each type were tested for their response in both assays.

RESULTS

Ozone alters plumes of C. pepo *and reacts with individual compounds*

Ozone alters the plume of *C. pepo.* When the plume of four *C. pepo* flowers was mixed with 120 ppbv ozone in a 2l bottle, the plume was dramatically different than when the plume was mixed with scrubbed air. Matching our expectations, indole and many other compounds were reactive with ozone, showing a decrease in abundance in the post-ozonation plume (Fig1A). 124TMB on the other hand was not reactive. When both compounds were tested in isolation, the same trend was observed: indole mixed with ozone showed a sharp decline in abundance, while 124TMB mixed with ozone for the same amount of time did not show a significant decline in abundance.

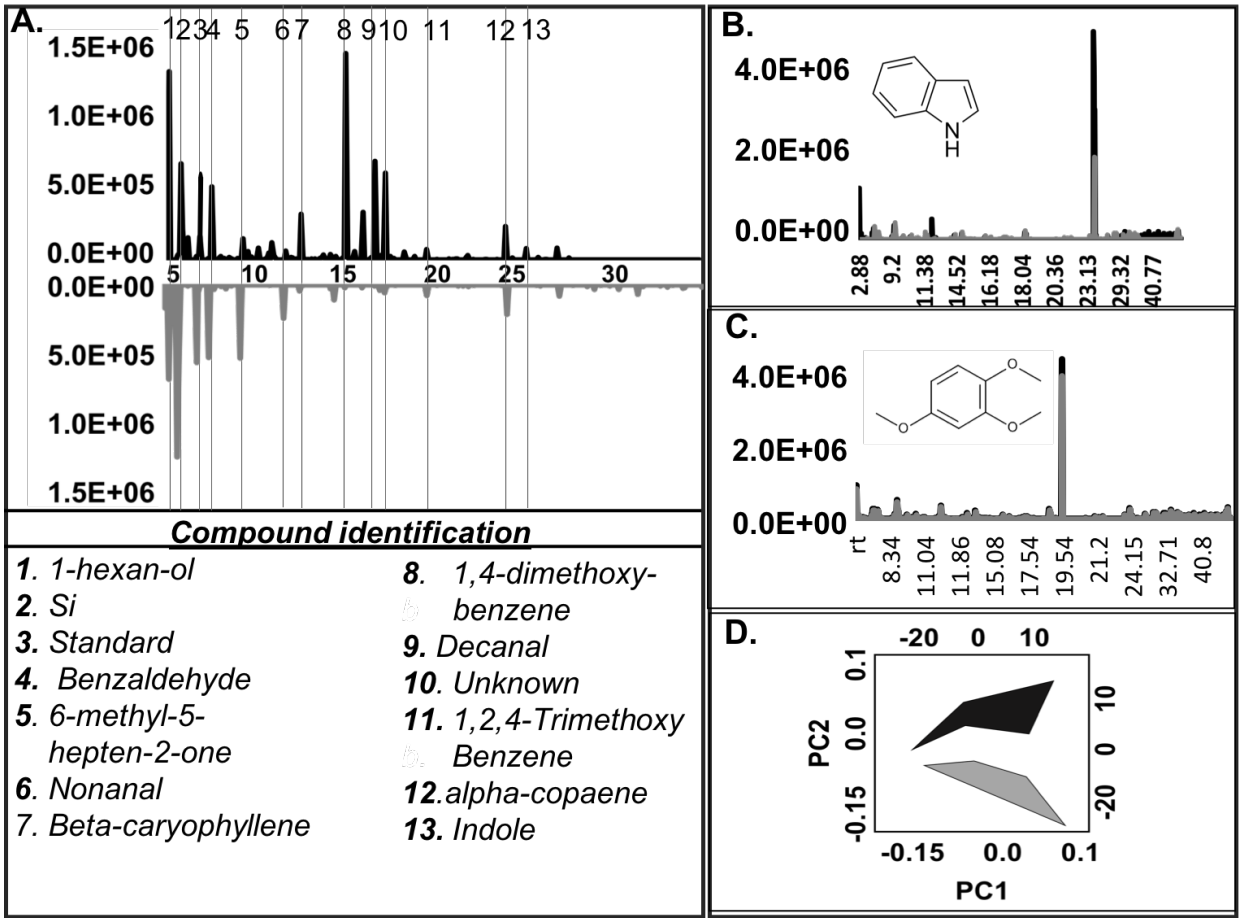


Fig. 1. *C. pepo* plumes are altered by ozone. **A.** Example traces of original (black) and ozonized (grey) headspaces of two *C. pepo* flowers. Numbered peaks are identified by the NIST library (R-match > 90%). **B.** Sample trace of indole mixed with air (black) compared to indole mixed with 120 ppbv of ozone (grey). Indole mixed with ozone was significantly reduced in abundance (paired t-test: p-value=0.0031, n=6) **C.** 124TMB mixed with air (black) and with ozone (grey) in the same manner did not show a significant decrease in abundance after mixing with ozone (paired t-test: p-value=0.7811, n=6**) D**. Principal component analysis of original and ozonized headspaces of five *C. pepo* flowers: air-exposed principal components are filled-in in black, and ozone-exposed principal components are filled in with grey.

Behavior assays

Response to individual plume components

Neither specialist nor generalist beetles were attracted or repulsed by the 80% hexane 20% acetone solvent mixture used to dilute key compounds, not did beetles distinguish between ozone and clean air (Supplementary Fig 1).The specialist found both single compounds (indole and 124TMB) attractive, while the generalist responded only to indole. When choosing between indole (reactive with ozone) mixed with air and indole mixed with ozone, both beetles preferred the single compound in air. However, *A. vittatum* did not distinguish between the compound 124TMB (unreactive with ozone) mixed in air or in ozone, maintaining its attraction to 124TMB despite the presence of ozone.

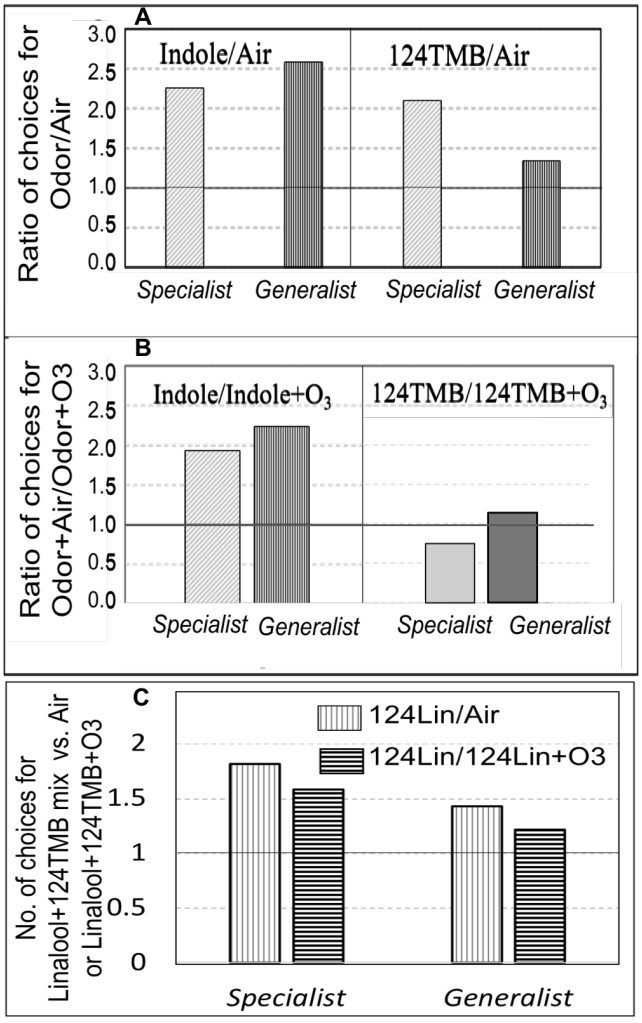


Fig. 2. Beetles respond to individual floral volatiles of *C. pepo* plume relative to air, and to individual compounds mixed with air vs. mixed with 120 ppbv ozone. **A.** Specialists prefered both indole (χ^2 = 7.53, p-value 0.006, n=25) and 124TMB (χ^2 = 10.56, p-value 0.0067, n=25) to clean air, while the generalist prefered indole (χ^2 = 6.76, p-value 0.0093, n=26) but not 124TMB (χ^2 =.3158, p-value 0.4531, n=20) to clean air. **B.** Both specialists and generalists preferred indole in air to indole in ozone (The specialist: χ^2 = 4.16, p-value 0.04109, n=34; The generalist χ^2 = 5.16, p-value 0.02314, n=30). Neither specialists nor generalists preferred 124TMB in air to 124TMB in ozone (The specialist: χ^2 = 0.143, p-value 0.7055, n=28; The generalist χ^2 = 0.111, p-value 0.7389, n=36) **C.** The specialist prefered 124TMB+Linalool to air (specialist: χ^2 = 5.76, p=value =0.016, n=33), while the generalist did not (χ^2 = 0.50, p=value =0.4795, n=33). Specialists prefered 124TMB+Linalool in air to 124TMB+Linalool in ozone (specialist: χ^2 = 3.98, p-value =0.038, n=22) while the generalist did not (specialist: χ^2 = 0.78, p-value=0.429, n=23).

Initial and learned response to the ozonated *C. pepo* plume and single compound

However, the specialist found the ozone-altered plume to be less attractive than the unaltered plume. When given the choice between the plume of *C. pepo* and air, both the generalist and the specialist beetle preferred the *C. pepo* plume, but when the plume was mixed with ozone, only the generalist continued to prefer it to air.

After beetles had fed in an ozone-enriched environment, the specialist's response to the ozonated plume changed. The specialist was able to learn the ozone-altered plume after feeding on *C. pepo* in an ozone-enriched environment. While the generalist maintained attraction to the ozone-altered plume both before and after training, the specialist significantly increased its preference for the ozone-altered plume after training. Beetles were further tested for their ability

to associatively learn the single floral compound linalool: before training, beetles did not distinguish linalool from air, and attraction to linalool did not increase after feeding on linalool-scented sugar solution.

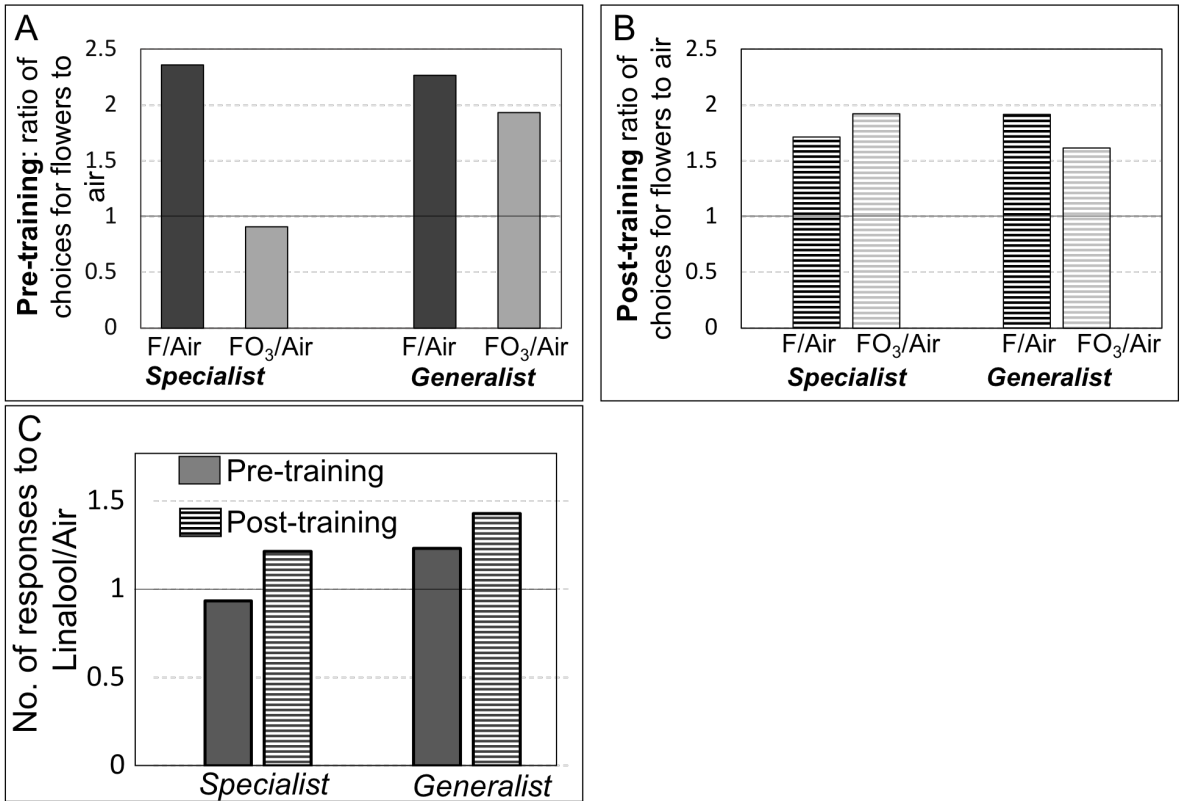


Fig. 3. The ratio of beetles choices for the *C. pepo* plume or the *C. pepo* plumed mixed with ozone to the choices for clean air. **A. Pre-training** Both beetles chose the pure plume more often than they chose scrubbed air (The specialist: χ^2=9.24, p-value=0.0020, n=46; The generalist χ^2=8.0, p-value=0.004, n=47). In contrast, the specialist does not chose the ozone-altered plume more often than air (χ^2 = .818, p-value= 0.3657, n=42) but the generalist does (χ^2 = 6.095, p-value 0.0136, n=45) **B. Post-training** Both beetles still prefered the pure plume to air (the specialist: χ^2 = 5.81 p-value =.0159, n=38; the generalist: χ^2 = 5.81 p-value =.0159,n=41) post-training, and furthermore, the specialist as well as the generalist prefered ozone-altered plumes to air (the specialist: χ^2 = 4.83, p-value=.0433, n=41; the generalist: χ^2 =4.455, p-value=.0348,

n=40). **C.** Beetles did not initially find linalool more attractive than air (the specialist: χ^2 =0.02 p-value=0.882 n=24; the generalist χ^2 =0.25 p-value=0.612, n=22), and failed to find linalool more attractive after feeding for at least 24 hours on linalool-scented sugar water (the specialist: χ^2=1.125, p-value=0.288; the generalist χ^2=1.05, p-value=0.3035).

DISCUSSION

C. pepo *plumes react with ozone*

Matching our predictions, the plume of *C. pepo* was greatly altered by ozonation, and the reactive compound indole was reduced in abundance after mixing with ozone, while the unreactive compound 124TMB was not (Fig.1A). This result was corroborated in individual tests of 124TMB and indole mixed with ozone (Fig.1B). Moreover, the volatile profile of the pure plume from the cultivar 'crookneck squash' used in this study was similar to that from published data on other cultivars, with major peaks of 1,4-dimethoxybenzene, as well as smaller amounts of indole and 1,2,4-trimethoxybenzene (Theis et al. 2009; Ferrari et al. 2006; Granero et al. 2005; Shapiro et al. 2001). When the plume of *C. pepo* was exposed to ozone, more compounds than just indole were reactive with ozone: five of the eight identified floral volatiles were reduced after exposure to ozone (Fig.1A). Furthermore, in addition to decreases in some compounds, ozonation also altered the ratios of compounds in the blend, changing a potentially meaningful part of plume composition for foraging insects (Bruce et al. 2005). Ratios in the blend were altered as some compounds became less abundant via reaction with ozone, while other compounds were not reactive, including benzaldehyde and 124TMB.

Specialist response to individual compounds aligns with predictions

Following our predictions that the specialist would be attracted to previously identified (Lewis et al. 1990; Adler et al. 2009) key compounds, we found that the specialist preferred both indole and 124TMB to air. This is in contrast to the generalist, which found only indole attractive (Fig.2). The generalist's attraction to indole, which is contradictory to our hypothesis that the generalist would not find any single compound attractive, has been demonstrated in some previous studies (Lampman et al. 1987) but not in others (Andersen and Metcalf 1986). Furthermore, the generalist's attraction to indole can be lost or gained throughout a field season (Lampman et al. 1987), indicating that the generalist beetle's preference for indole may be based on their foraging experiences.

Both beetles' reactions to the single compounds mixed with ozone matched with our predictions: when given the choice between the reactive compound indole mixed with ozone or air, both beetles preferred indole mixed with air, likely preferring the higher concentration of indole in the scrubbed air treatment or potentially discriminating against breakdown products that would be present in the ozone-mixed indole plume. Conversely, when the specialist was given the choice between the unreactive compound 124TMB mixed with air or mixed with ozone, it did not demonstrate a preference for one or the other. This demonstrates that the ozone-reactivity of a compound can determine its attractiveness in ozone-enriched environments.

Generalist and specialist response to the ozonated plume

Do generalist and specialist beetles' responses to an ozonated floral plume differ, and is at least one unreacted key compound sufficient to attract a specialist to an ozonated floral plume if other key compounds are reactive? First, we tested beetle attraction to the unaltered plume of *C. pepo*, determining that both beetles found the plume attractive (Fig.3A). However, after the plume was exposed to ozone, the specialist, but not the generalist, lost this attraction (Fig.3B). Because

ozone by itself is not a deterrent to either beetle (Supplementary Fig. 1), the plume of *C. pepo* must be altered in such a way that it is no longer attractive to the foraging specialist. The specialist's loss of attraction to the ozonated plume despite the presence of the attractive compound 124TMB is in contrast to our predictions, given our findings that the single compound 124TMB presented alone in ozone was sufficient to attract the specialist (Fig.2A). The specialist's loss of attraction to the ozonated plume indicates that even in the 'best case scenario' where a key compound remains unreacted in the ozonated plume, a specialist may still lose attraction to ozonated plumes. One possible mechanistic explanation for the loss of attraction to the ozone-exposed plume despite the presence of an attractive compound is that secondary products resulting from ozonation of other reactive compounds in the plume may deter the specialist, as has been suggested in previous work (Li et al. 2016). This is further supported by the specialist's decreased attraction to the ozonated mixture of 124TMB and linalool relative to the beetle's response to 124TMB and linalool in air. The beetle's response to the mixture of 124TMB and linalool was not directly compared to its response to 124TMB by itself, but the beetle's response to both odors (124TMB and 124TMB + linalool) was compared to air: specialist beetles chose 124TMB by itself vs. air at a rate of 2.1:1 (Fig.2A), while choosing 124TMB+linalool vs. air at a rate of 1.78:1 (Fig.2C), indicating that the addition of linalool did not increase the attractiveness of 124TMB. Moreover, linalool by itself was not an attractant (Fig.3C). However, the mixture of 124TMB and linalool became less attractive to the specialist after exposure to ozone (Fig.2C), indicating that reaction products of linalool and ozone, rather than a decrease in concentration of linalool, negatively impacted the specialist's response to the two-compound blend.

The specialist's loss of attraction to the ozone-altered *C. pepo* plume, despite its maintained attraction to 124TMB, paired with the generalist's maintained attraction to the *C.*

pepo plume, indicates that specialists may be at greater risk of ozone-plume disturbance than generalists. The generalist maintained its attraction to the ozone-altered plume because some subset of important floral volatiles attractive to the generalist were not detectably altered by ozone. The generalist's attraction to the ozone-altered plume may reflect the wide range of potentially attractive cues that a generalist can utilize and which enable a generalist to forage on taxonomically diverse host plants. In contrast, the specialist, which lost its attraction to the ozonated plume, may be deterred if alterations to the plume, such as the addition of secondary compounds or the loss of distinctive *Cucurbita* compounds, change the insect's recognition of the flower as a cucurbit, a stringent criterion that a generalist would not have to meet. Thus, the beetles' responses to ozone-exposed plumes may be indicative of responses for generalist and specialist insects more broadly.

Learning as a means of coping with ozone-altered plumes

The specialist's loss of attraction to the ozone-altered *C. pepo* plume provides troubling evidence of the negative impact of ozone on insect foraging success, particularly for specialist insects. However, the specialist was able to improve upon its initial disinterest in the ozone-altered *C. pepo* plume after associating the altered plumes with food rewards (Fig.3C). The specialist's ability to learn new olfactory signals is consistent with studies demonstrating olfactory-cue learning in some specialist parasitoid species (Turlings et al. 1993; Stiedle et al. 1997; Potting et al. 1999). While the specialist was not capable of learning the single odorant linalool after feeding on linalool-scented sugar water, the generalist was not capable of learning linalool in this manner either, likely reflecting the challenge of learning a single compound, although it is possible that the beetles could not detect linalool. Linalool was selected as it is readily learned by some pollinating bees (e.g. Laloi et al 1999; Laloi and Pham-Delegue 2004; Sommerland et al

2014), but the compounds that are easy to learn for a pollinator may not be the same ones readily learned by an herbivore, even assuming that herbivores have a wide capacity to learn olfactory cues. Learning olfactory cues may help specialist insects select the best possible host when presented with several options, considering that the variation in olfactory signals (as well as nutritional quality) from taxonomically similar plants, including those from a single genus, is still substantial (e.g. Dobson et al. 1997). Moreover, particularly pertinent to the specialist investigated here, *Cucurbita* species and even varieties of the same *Cucurbita* species show a range in composition of floral compounds (Theis et al. 2009; Ferrari et al. 2006; Granero et al. 2005; Shapiro et al. 2001). While the ability to learn an ozone-altered plume of a host plant has already been documented (Cook et al in prep.), this is the first study which finds that a genus-specific specialist can learn the initially non-attractive ozone-altered plume of its host plant. The specialist's ability to learn the ozone-altered plume indicates that learning may be a strategy employed by specialists as well as generalists and olfactory learning may help specialists to cope with rapidly changing scentscapes induced by increased loads of tropospheric oxidants.

Risks for generalists vs. specialists in polluted areas

Despite the specialist's surprising ability to learn ozone-perturbed plumes, specialist insects generally may still be at greater risk than generalists in ozone-perturbed scentscapes. While generalist insects unable to locate a preferred host plant may simply switch onto less-preferred host plant options, specialist insects unable to locate their limited host plants could face starvation. At best, an individual specialist's innate response to ozone-altered plumes will depend on whether the olfactory compounds it uses are reactive with ozone; given the small number of those 'token' compounds and the fact that most floral volatiles currently examined are reactive with ozone (Atkinson and Arey 2003), many naïve specialist insects are likely to emerge to find

that the innately-attractive olfactory cues they rely on to locate host plants are not proximally available in ozone-enriched environments (unless specialists have specifically selected for oxidant-resistant olfactory cues). Furthermore, this study finds that even in the case where a specialist maintained attraction to a single-compound in ozone, this attraction was not sufficient to make the ozone-altered plume attractive before training on the ozonated plume, so even in scenarios where not all token compounds a specialist uses are reactive with ozone, air pollution could still perniciously impact specialist insects' foraging.

Moreover, while learning offers a potential strategy for coping with plume perturbations, it also has limitations. In order to learn olfactory cues, an insect must first locate its host plant relying on innate visual or olfactory cues. This may be particularly challenging for specialist species that forage solely on agricultural crops, such as the specialist tested here, the striped cucumber beetle *A. vittatum,* and the important pollinating squash bee *Peponapis pruinosa*. These insects do not have native host plants available in regions of the Eastern US, and instead they depend on squash crops for sustenance. These insects lay eggs for their young in the soil at the base of squash plants, and so initial naive-foraging insects can emerge directly into squash plants, eliminating the need for an initial long-distance host location. However, due to crop rotation or other human-determined causes, squash plants may be removed from the areas where squash bees and cucumber beetles overwinter, and naïve insects may emerge far from their host plants. In high ozone environments, this could mean emerging without intact olfactory cues to direct these insects to their host plants.

Furthermore, learning ozone-exposed plumes also requires an insect to be exposed to ozone while foraging. As a strong oxidant, ozone can perniciously impact insect health (Alstad et al. 1982) and may even damage insect antennae and thus the insect's ability to detect olfactory cues. In this study, both specialist and generalist ozone-exposed beetles responded less

frequently to pure plumes after ozone-exposure relative to beetles that had not been exposed to ozone for training (Fig.3A; Fig.3B). While this response was not significant, the shared directionality of response for specialists and generalists exposed to ozone indicates that ozone may affect perception, and shows an important line of questions for future studies.

Broader impacts and future research

Insects have experienced alarming worldwide declines in recent decades (Potts et al. 2010; Kluser et al. 2007; Hallman et al. 2018; Sanchez-Bayo and Wyckhuys 2019). While many anthropogenic drivers have been cited as the cause of these declines, anthropogenically driven air pollution, which manifests on a global scale, could be a contributing stressor. We find that ozone-altered plumes of a *Cucurbita pepo* host plant are not attractive to a specialist forager despite its attraction to an unreactive compound in the plume. In contrast to the specialist's loss of attraction, the generalist maintained its attraction to the ozone-altered plume, indicating that specialists in ozone-enriched environments may be at higher risk of losing recognition of floral plumes of their host plants compared to generalists. Furthermore, these results may be even more dramatic in work testing the response of naïve insects without experience in the field. Specialists are not without recourse in altered scentscapes however: a specialist was able to learn the ozone-altered plume of one of its host plants. This learning ability, while it has limitations, could enable foraging specialists to somewhat mitigate the impacts of ozone-induced alterations to floral plumes. Future research should investigate both the drivers of specialist attraction to ozonated floral plumes and the impacts of ozonated plumes on naïve and experienced insects, considering them as active agents capable of adapting behaviorally to their environments.

Citations

Adler, L. S., & Hazzard, R. V. (2009). Comparison of perimeter trap crop varieties: effects on herbivory, pollination, and yield in butternut squash. *Environmental entomology*, *38*(1), 207-215.

Ahmad, S. (Ed.). (2012). *Herbivorous insects: host-seeking behavior and mechanisms*. Elsevier.

Andersen, J. F., & Metcalf, R. L. (1986). Identification of a volatile attractant for Diabrotica and Acalymma spp. from blossoms of Cucurbita maxima Duchesne. *Journal of chemical ecology*, *12*(3), 687-699

Andrews, E. S., Theis, N., & Adler, L. S. (2007). Pollinator and herbivore attraction to Cucurbita floral volatiles. *Journal of Chemical Ecology*, *33*(9), 1682-1691

Atkinson, R., & Arey, J. (2003). Gas-phase tropospheric chemistry of biogenic volatile organic compounds: a review. *Atmospheric Environment*, *37*, 197-219.

Awano, K., Ichikawa, Y., Tokuda, K., & Kuraoka, M. (1997). Volatile components of the flowers of two Calanthe species. *Flavour and fragrance journal*, *12*(5), 327-330.

Baker J., Arey, J., & Atkinson, R. (2004). Kinetics of the gas-phase reactions of OH radicals, NO3 radicals and O3

Bell, W. J. (1990). Searching behavior patterns in insects. *Annual review of entomology*, *35*(1), 447-467.

Bernays, E. A., & Chapman, R. E. (1994). Behavior: the process of host-plant selection. *Host-plant selection by phytophagous insects*, 95-165.

Brandt, K., Dötterl, S., Francke, W., Ayasse, M., & Milet-Pinheiro, P. (2016). Flower Visitors of Campanula: Are Oligoleges More Sensitive to Host-Specific Floral Scents Than Polyleges?. *Journal of Chemical Ecology*, 1-9.

Blight, M. M., Pickett, J. A., Wadhams, L. J., & Woodcock, C. M. (1995). Antennal perception of oilseed rape, Brassica napus (Brassicaceae), volatiles by the cabbage seed weevil Ceutorhynchus assimilis (Coleoptera, Curculionidae). *Journal of chemical ecology*, *21*(11), 1649-1664.

Bruce, T. J., Wadhams, L. J., & Woodcock, C. M. (2005). Insect host location: a volatile situation. *Trends in plant science*, *10*(6), 269-274.

Bruce, T. J., & Pickett, J. A. (2011). Perception of plant volatile blends by herbivorous insects–finding the right mix. *Phytochemistry*, *72*(13), 1605-1611.

Brust, G. E., & House, G. J. (1990). Effects of soil moisture, no-tillage and predators on southern corn rootworm (Diabrotica undecimpunctata howardi) survival in corn agroecosystems. *Agriculture, Ecosystems & Environment*, *31*(3), 199-215.

Brasseur, G. P., Muller, J. F., Tie, X., & Horowitz, L. (2001). Tropospheric ozone and climate: past, present and future. *Present and Future of Modeling Global Environmental Change: Toward Integrated Modeling. TERRAPUB*, 63-75.

Burger, Hannah, Stefan Dötterl, and Manfred Ayasse. "Host-plant finding and recognition by visual and olfactory floral cues in an oligolectic bee." *Functional Ecology* 24.6 (2010): 1234-1240.

Burger H, Do¨tterl S, Ayasse M (2010b) Host plant finding and recognition by visual and olfactory floral cues in an oligolectic bee. Funct Ecol 24:1234 1240

Costa, A., & Reeve, J. D. (2011). Olfactory experience modifies semiochemical responses in a bark beetle predator. *Journal of chemical ecology*, *37*(11), 1166-1176.

Davis, S. C. (1997). Transportation Energy Data Book: Edition 17. ORNL-6919. Oak Ridge, TN: Oak Ridge National Laboratory.

De Jong, R., & Kaiser, L. (1991). Odor learning by Leptopilina boulardi, a specialist parasitoid (Hymenoptera: Eucoilidae). *Journal of Insect Behavior*, *4*(6), 743-750.
Dobson, H. E., Arroyo, J., Bergström, G., & Groth, I. (1997). Interspecific variation in floral fragrances within the genus Narcissus (Amaryllidaceae). *Biochemical Systematics and Ecology*, *25*(8), 685-706.
Dudareva, N., & Pichersky, E. (Eds.). (2006). *Biology of floral scent*. CRC press.
Ellers-Kirk, C., & Fleischer, S. J. (2006). Development and life table of Acalymma vittatum (Coleoptera: Chrysomelidae), a vector of Erwinia tracheiphila in cucurbits. *Environmental Entomology*, *35*(4), 875-880.
Eltz, T., Whitten, W. M., Roubik, D. W., & Linsenmair, K. E. (1999). Fragrance collection, storage, and accumulation by individual male orchid bees. *Journal of Chemical Ecology*, *25*(1), 157-176.
Effmert, U., Dinse, C., & Piechulla, B. (2008). Influence of green leaf herbivory by Manduca sexta on floral volatile emission by Nicotiana suaveolens. *Plant physiology*, *146*(4), 1996-2007
Farré-Armengol, G., Peñuelas, J., Li, T., Yli-Pirilä, P., Filella, I., Llusia, J., & Blande, J. D. (2016). Ozone degrades floral scent and reduces pollinator attraction to flowers. *New Phytologist*, *209*(1), 152-160.
Ferrari, M. J., Stephenson, A. G., Mescher, M. C., & De Moraes, C. M. (2006). Inbreeding effects on blossom volatiles in Cucurbita pepo subsp. texana (Cucurbitaceae). *American Journal of Botany*, *93*(12), 1768-1774.
Fiore, A. M., Jacob, D. J., Bey, I., Yantosca, R. M., Field, B. D., Fusco, A. C., & Wilkinson, J. G. (2002). Background ozone over the United States in summer: Origin, trend, and contribution to pollution episodes. *Journal of Geophysical Research: Atmospheres*, *107*(D15), ACH-11.
Fraenkel, G. S. (1959). The raison d'etre of secondary plant substances. *Science*, 1466-1470.
Fuentes, J. D., T'ai, H. R., & Zenker, J. (2013). Ozone impedes the ability of a herbivore to find its host. *Environmental Research Letters*, *8*(1), 014048.
Granero, A. M., Gonzalez, F. E., Sanz, J. G., & Vidal, J. M. (2005). Analysis of biogenic volatile organic compounds in zucchini flowers: identification of scent sources. *Journal of chemical ecology*, *31*(10), 2309-2322.
Galen, C., Kaczorowski, R., Todd, S. L., Geib, J., & Raguso, R. A. (2011). Dosage-dependent impacts of a floral volatile compound on pollinators, larcenists, and the potential for floral evolution in the alpine skypilot Polemonium viscosum. *The American Naturalist*, *177*(2), 258-272.
Hammer, M., & Menzel, R. (1995). Learning and memory in the honeybee. *Journal of Neuroscience*, *15*(3), 1617-1630.
Hansson, BilL S., Mattias C. Larsson, and Walter S. Leal. Green leaf volatile-detecting olfactory receptor neurones display very high sensitivity and specificity in a scarab beetle. *Physiological Entomology* 24.2 (1999): 121-126.
Hoffmann, M. P., Kirkwyland, J. J., Smith, R. F., & Long, R. F. (1996). Field Tests with Kairomone–Baited Traps for Cucumber Beetles and Corn Rootworms in Cucurbits. *Environmental entomology*, *25*(5), 1173-1181.
Irwin RE, Adler LS, Brody AK (2004) The dual role of floral traits: pollinator attraction and plant defense. *Ecology* 85:1503–1511
Jacob, D. J. (1999). *Introduction to atmospheric chemistry*. Princeton University Press.

Jürgens, Andreas, Taina Witt, and Gerhard Gottsberger. Flower scent composition in Dianthus and Saponaria species (Caryophyllaceae) and its relevance for pollination biology and taxonomy. *Biochemical Systematics and Ecology* 31.4 (2003): 345-357.

Knauer, A. C., & Schiestl, F. P. (2017). The effect of pollinators and herbivores on selection for floral signals: a case study in Brassica rapa. *Evolutionary ecology*, *31*(2), 285-304.

Knudsen, J. T. (1994). Floral scent variation in the Pyrola rotundifolia complex in Scandinavia and western Greenland. *Nordic Journal of Botany*, *14*(3), 277-282.

Knudsen, J. T., Tollsten, L., & Bergström, L. G. (1993). Floral scents—a checklist of volatile compounds isolated by head-space techniques. *Phytochemistry*, *33*(2), 253-280.

Krysan, J. L. (1986). Introduction: biology, distribution, and identification of pest Diabrotica. In *Methods for the study of pest Diabrotica* (pp. 1-23). Springer, New York, NY.

Kunze, J., & Gumbert, A. (2001). The combined effect of color and odor on flower choice behavior of bumble bees in flower mimicry systems. *Behavioral Ecology*, *12*(4), 447-456.

Laloi, D., Roger, B., Blight, M. M., Wadhams, L. J., & Pham-Delegue, M. H. (1999). Individual learning ability and complex odor recognition in the honey bee, Apis mellifera L. *Journal of insect behavior*, *12*(5), 585-597

Laloi, D., Pham-Delègue, M.H. (2004) Bumblebees show asymmetrical discrimination between two odors in a classical conditioning procedure. J. Insect Behav. 17(3), 385–396

Lampman, R. L., Metcalf, R. L., & Andersen, J. F. (1987). Semiochemical attractants of Diabrotica undecimpunctata howardi Barber, southern corn rootworm, and Diabrotica virgifera virgifera LeConte, the western corn rootworm (Coleoptera: Chrysomelidae). *Journal of chemical ecology*, *13*(4), 959-975

Lampman, R. L., & Metcalf, R. L. (1990). Kairomonal attractants for Acalymma vittatum (Coleoptera: Chrysomelidae). *Environmental entomology*, *19*(1), 8-14

Lee, A., Goldstein, A. H., Keywood, M. D., Gao, S., Varutbangkul, V., Bahreini, R., ... & Seinfeld, J. H. (2006). Gas-phase products and secondary aerosol yields from the ozonolysis of ten different terpenes. *Journal of Geophysical Research: Atmospheres*, *111*(D7).

Lewis, P. A., Lampman, R. L., & Metcalf, R. L. (1990). Kairomonal attractants for Acalymma vittatum (Coleoptera: Chrysomelidae). *Environmental entomology*, *19*(1), 8-14

Li, T., Blande, J. D., & Holopainen, J. K. (2016). Atmospheric transformation of plant volatiles disrupts host plant finding. *Scientific Reports*, *6*, 33851.

Lusebrink, I., Girling, R. D., Farthing, E., Newman, T. A., Jackson, C. W., & Poppy, G. M. (2015). The effects of diesel exhaust pollution on floral volatiles and the consequences for honey bee olfaction. *Journal of chemical ecology*, *41*(10), 904-912.

Metcalf, R. L., & Lampman, R. L. (1989). The chemical ecology of Diabroticites and Cucurbitaceae. *Experientia*, *45*(3), 240-247.

Metcalf, R. L., & Lampman, R. L. (1991). Evolution of diabroticite rootworm beetle (Chrysomelidae) receptors for Cucurbita blossom volatiles. *Proceedings of the National Academy of Sciences*, *88*(5), 1869-1872.

Metcalf, R. L., Lampman, R. L., & Deem-Dickson, L. (1995). Indole as an olfactory synergist for volatile kairomones for diabroticite beetles. *Journal of chemical ecology*, *21*(8), 1149-1162

McFrederick, Q. S., Kathilankal, J. C., & Fuentes, J. D. (2008). Air pollution modifies floral scent trails. *Atmospheric Environment*, *42*(10), 2336-2348.

McFrederick, Q. S., Fuentes, J. D., Roulston, T. A., Kathilankal, J. C., & Lerdau, M. (2009). Effects of air pollution on biogenic volatiles and ecological interactions. *Oecologia*, *160*(3), 411-420.

Milet-Pinheiro, P., Ayasse, M., Schlindwein, C., Dobson, H. E., & Dötterl, S. (2012). Host location by visual and olfactory floral cues in an oligolectic bee: innate and learned behavior. *Behavioral Ecology*, *23*(3), 531-538.

Natale, D., Mattiacci, L., Hern, A., Pasqualini, E., & Dorn, S. (2003). Response of female Cydia molesta (Lepidoptera: Tortricidae) to plant derived volatiles. *Bulletin of entomological research*, *93*(4), 335-342.

Nottingham, S. F., Hardie, J., Dawson, G. W., Hick, A. J., Pickett, J. A., Wadhams, L. J., & Woodcock, C. M. (1991). Behavioral and electrophysiological responses of aphids to host and nonhost plant volatiles. *Journal of chemical ecology*, *17*(6), 1231-1242.

Orlando, J. J., Nozière, B., Tyndall, G. S., Orzechowska, G. E., Paulson, S. E., & Rudich, Y. (2000). Product studies of the OH-and ozone-initiated oxidation of some monoterpenes. *Journal of Geophysical Research: Atmospheres*, *105*(D9), 11561-11572.

Papaj, D. R., & Prokopy, R. J. (1989). Ecological and evolutionary aspects of learning in phytophagous insects. *Annual review of entomology*, *34*(1), 315-350.

Potting, R. P. J., Poppy, G. M., & Schuler, T. H. (1999). The role of volatiles from cruciferous plants and pre-flight experience in the foraging behaviour of the specialist parasitoid Cotesia plutellae. *Entomologia experimentalis et applicata*, *93*(1), 87-95.

Prokopy, R. J., & Owens, E. D. (1983). Visual detection of plants by herbivorous insects. *Annual review of entomology*, *28*(1), 337-364.

Reeves, J. L. (2011). Vision should not be overlooked as an important sensory modality for finding host plants. *Environmental Entomology*, *40*(4), 855-863.

Riffell, J. A., Lei, H., Abrell, L., & Hildebrand, J. G. (2013). Neural basis of a pollinator's buffet: olfactory specialization and learning in Manduca sexta. *Science*, *339*(6116), 200-204

Riveros, A. J., & Gronenberg, W. (2009). Olfactory learning and memory in the bumblebee Bombus occidentalis. *Naturwissenschaften*, *96*(7), 851-856.

Ryerson, T. B., Trainer, M., Holloway, J. S., Parrish, D. D., Huey, L. G., Sueper, D. T., ... & Kuster, W. C. (2001). Observations of ozone formation in power plant plumes and implications for ozone control strategies. *Science*, *292*(5517), 719-723.

Sánchez-Bayo, F., & Wyckhuys, K. A. (2019). Worldwide decline of the entomofauna: A review of its drivers. Biological Conservation, 232, 8-27.

Sandermann, H., Ernst, D., Heller, W., & Langebartels, C. (1998). Ozone: an abiotic elicitor of plant defence reactions. *Trends in Plant Science*, *3*(2), 47-50.

Schäffler, I., Steiner, K. E., Haid, M., van Berkel, S. S., Gerlach, G., Johnson, S. D., ... & Dötterl, S. (2015). Diacetin, a reliable cue and private communication channel in a specialized pollination system. *Scientific reports*,*5*, 12779.

Schiestl FP, Huber FK, Gomez JM (2011) Phenotypic selection on floral scent: trade-off between attraction and deterrence? Evolutionary Ecology 25:237–248

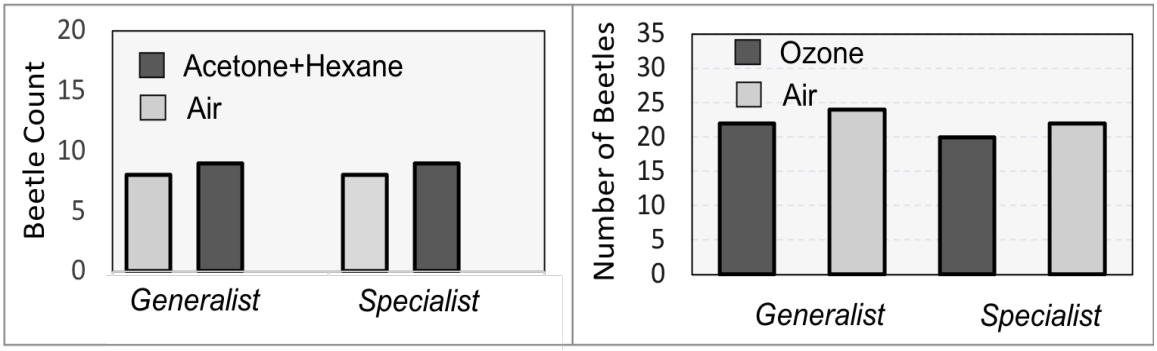
Schnell, R. C., Oltmans, S. J., Neely, R. R., Endres, M. S., Molenar, J. V., & White, A. B. (2009). Rapid photochemical production of ozone at high concentrations in a rural site during winter. *Nature Geoscience*, *2*(2), 120.

Simões, P., Ott, S. R., & Niven, J. E. (2011). Associative olfactory learning in the desert locust, Schistocerca gregaria. *Journal of Experimental Biology*, *214*(15), 2495-2503.

Sommerlandt, F. M., Rössler, W., & Spaethe, J. (2014). Elemental and non-elemental olfactory learning using PER conditioning in the bumblebee, Bombus terrestris. *Apidologie*, *45*(1), 106-115.
Steidle, J. L., & Schöller, M. (1997). Olfactory host location and learning in the granary weevil parasitoid Lariophagus distinguendus (Hymenoptera: Pteromalidae). *Journal of insect behavior*, *10*(3), 331-342.
Stenberg, J. A., & Ericson, L. (2007). Visual cues override olfactory cues in the host-finding process of the monophagous leaf beetle *Altica engstroemi*. *Entomologia Experimentalis et Applicata*, *125*(1), 81-88.
Svensson, G. P., Hickman Jr, M. O., Bartram, S., Boland, W., Pellmyr, O., & Raguso, R. A. (2005). Chemistry and geographic variation of floral scent in Yucca filamentosa (Agavaceae). *American Journal of Botany*, *92*(10), 1624-1631
Tallamy, D. W., & Gorski, P. M. (1997). Long-and short-term effect of cucurbitacin consumption on Acalymma vittatum (Coleoptera: Chrysomelidae) fitness. *Environmental entomology*, *26*(3), 672-677.
Theis N (2006) Fragrance of Canada thistle (*Cirsium arvense*) attracts both floral herbivores and pollinators. J Chem Ecol 32:917–927
Theis, N., & Adler, L. S. (2012). Advertising to the enemy: enhanced floral fragrance increases beetle attraction and reduces plant reproduction. *Ecology*, *93*(2), 430-435.
Theis, N., Kesler, K., & Adler, L. S. (2009). Leaf herbivory increases floral fragrance in male but not female Cucurbita pepo subsp. texana (Cucurbitaceae) flowers. *American journal of botany*, *96*(5), 897-903. (this one doesn't look very similar)
Turlings, T. C., Wäckers, F. L., Vet, L. E., Lewis, W. J., & Tumlinson, J. H. (1993). Learning of host-finding cues by hymenopterous parasitoids. In *Insect learning* (pp. 51-78). Springer, Boston, MA.
Vet, L. E., Lewis, W. J., & Carde, R. T. (1995). Parasitoid foraging and learning. In *Chemical ecology of insects 2* (pp. 65-101). Springer, Boston, MA.
Vingarzan, R. (2004). A review of surface ozone background levels and trends. *Atmospheric environment*, *38*(21), 3431-3442.
Visser, J. H., and D. A. Avé. "General green leaf volatiles in the olfactory orientation of the Colorado beetle, Leptinotarsa decemlineata." *Entomologia experimentalis et applicata* 24, no. 3 (1978): 738-749.
Visser, J. H. (1986). Host odor perception in phytophagous insects. *Annual review of entomology*, *31*(1), 121-144.
Visser, J. H. (1988). Host-plant finding by insects: orientation, sensory input and search patterns. *Journal of insect physiology*, *34*(3), 259-268
Widjaja, R., Craske, J. D., & Wootton, M. (1996). Comparative studies on volatile components of non-fragrant and fragrant rices. *Journal of the Science of Food and Agriculture*, *70*(2), 151-161.
Young PJ, et al. (2013), Pre-industrial to end 21st century projections of tropospheric ozone from the Atmospheric Chemistry and Climate Model Intercomparison Project (ACCMIP). *Atmos. Chem. Phys* 13: 2063–2090.
Zanen, P. O., & Cardé, R. T. (1991). Learning and the role of host-specific volatiles during in-flight host-finding in the specialist parasitoid Microplitis croceipes. *Physiological entomology*, *16*(3), 381-389.

Supplementary Figure

Beetle response to hexane/acetone and air and ozone and air find that beetles do not discern between ozone and air, or the solvent mixture of acetone and hexane and air. Thirty beetles of each species (generalist and specialist) were tested for their response to either acetone+ hexane or air, but many made no choice when presented with these unattractive options. Likewise, 45 beetles of each species was tested for their response to ozone vs. air, and again many beetles did not make a choice for either gas presented.



Supplementary Fig. 1. Beetle's response to solvent vs. air or to ozone vs. air. **A.** Neither generalists nor specialist beetles preferred acetone+hexane to air (*The Generalist:* χ^2 *=0.124, p-value=0.768; The Specialist* χ^2 *=0.094, p-value=.868*). **B.** Neither the generalist (χ^2 *=0.041, p-value=0.847)* nor the specialist *(*χ^2 *=0.182, p-value = 0.669)* beetles discerned between ozone and air.

Chapter #4

Ozone alters floral signals and rewards, making plants less attractive to a specialist pollinator

Summary

Pollination is critical to agricultural and natural ecosystems, and it is maintained when plants produce honest signals that pollinators can recognize and correlate with rewards. However, plant-pollinator interactions may be jeopardized if elevated air pollution disrupts the production of floral signals and rewards. Here we find that elevated levels of the pollutant ozone can impact some floral signals and rewards for three taxonomically diverse plant species: *Petunia spp*., *Nicotiana alata*, and *Cucurbita pepo*, although ozone-induced changes were not consistent across the sampled plant taxa. Changes to floral signals and rewards have implications for pollinators' ability to locate and recognize host plants, as well as for their preferences for host plants. We tested the response of two pollinators to ozone-exposed and air-exposed *C. pepo* plants, finding that the specialist squash bee, *Peponapis pruinosa*, discriminates against ozone-exposed plants, while the European honeybee, *Apis mellifera,* does not. In addition to causing alterations in the production of floral signals and floral rewards, ozone may also disrupt pollinator attraction by reacting with floral plumes as they move downwind of the plant. Here we find that the plumes of *N. alata*, *C. pepo*, and petunia were all altered after exposure to ozone, and squash bees did not find the *C. pepo* floral plumes mixed with ozone attractive. From these findings, we conclude that atmospheric pollution has the potential to interfere with plant-pollinator interactions both by altering the production of floral signals and rewards that attract and reinforce pollinator choice and by interfering with floral signals post-production. Tropospheric ozone could thus have serious impacts on native ecosystems and managed agricultural systems that depend on insect pollination.

INTRODUCTION

Importance and definition of pollination

Organisms that reproduce sexually face a substantial hurdle to reproduction: locating a mate. This problem is exacerbated in the case of sessile organisms such as plant species. To overcome this obstacle to reproduction, many plants have evolved a mutualism with animals, wherein animals act as vectors the plant's genetic material. Plants recruit animal vectors by offering floral rewards, including both pollen, containing the genetic material, and nectar. Over 80% of flowering plants worldwide rely on animal pollinators, primarily insects, to reproduce (Ollerton et al. 2011). To attract pollinators, plants must advertise their rewards using visual cues (Giurfa and Lehrer 2001; Gould 1985) and/or olfactory cues, such as floral scents (Kunze and Gumbert 2001). Floral scents may play a particularly significant role in long-distance communication (e.g Dotterl & Schaffler 2007), as floral scents can travel beyond the range of visual cues.

Pollinators use floral signals and rewards to make choices about which plants to visit.

Pollen and nectar are the primary source of proteins and carbohydrates for many pollinators (Brodschneider & Crailsheim 2010), with bees using pollen as the sole source of protein for their larvae (Gilbert 1972; Roulston and Cane 2000). Given their importance as major food-sources, pollen and nectar play a role in pollinator floral choice. Pollinators can preferentially choose plants with high rewards via association of both visual (Gumbert et al. 2000) and olfactory (Wright and Schiestl 2009) cues with the quantity or quality of rewards proffered at the flower. If the overall desirability of the rewards is high, then the pollinator forms a positive association between the floral signals and rewards and can subsequently seek out other plants presenting the

same signals as the previously rewarding host. This act of floral constancy enforces cue honesty and benefits pollinators and plants alike, increasing insect efficiency at locating resources (Wilson and Stine 1996; Raine and Chittka 2011) and maximizing the transfer of pollen to conspecifics.

'Good' rewards, or rewards that are likely to lead to positive associations, are constituted by their abundance and nutritional quality, most rudimentarily described by the sugar concentration of nectar and the protein content of pollen. Numerous studies have documented pollinator preference for flowers with high nectar abundance (Waddington & Gottlieb 1990) and high nectar sugar content (Bitterman et al. 1976; Hill et al. 2001), and nectar that is abundant and has a high nectar sugar concentration is more readily learned than nectar that is less abundant or has a lower sugar concentration.(Buchanan & Bitterman 1988; Lee & Bitterman 1990; Couvillon & Bitterman 1993). Pollen also plays an important role in determining pollinator choice (Robertson et al. 1999; Muth et al. 2016).While the amount of pollen presented has not been found to be a strong driver of pollinator choice (Somme et al. 2015), previous work finds that pollinators prefer pollen with a high protein content (Vaudo et al. 2016; Konzmann and Lunau 2014; Cardoza et al. 2012; Hanley et al. 2008; Kitaoka et al. 2008). However, these studies do not disentangle pollen quality from signals correlated with pollen such as odor. Pollen odor has been demonstrated to motivate foraging activity (Kitaoka and Nieh 2008), attract insects (Dobson 1987; Dobson et al. 1996; Cook et al. 2002), and potentially act as plant-specific cues for specialist foragers (Linsley et al. 1958), and could thus be drivingpollinator's preference forhigh-protein. While pollen taste can inform pollinator choice (Muth et al. 2016), it is currently unclear if pollinators can recognize pollen quality by taste alone (Pernal and Curre 2002; Roulston, Cane, and Buchmann 2000). However, despite uncertainty in how pollinators discern

pollen quality, both pollen and nectar rewards do play a major role in influencing pollinator choice, with pollinators preferentially visiting more rewarding flowers after experience.

Anthropogenically elevated tropospheric oxidants can damage flowers

Pollinators' choice to visit a given flower is thus shaped by floral signals and rewards that attract, incentivize, and inform pollinators: however, these signals and rewards may be altered by anthropogenically elevated air pollution. As a result of increased fossil fuel combustion following the industrial revolution, atmospheric pollutants have increased dramatically (Vingarzan 2004). Perhaps most concerning to plant species is the increase in tropospheric oxidants, including the tropospheric oxidant ozone. Tropospheric ozone has increased from worldwide concentrations of approximately 10 ppbv in pre-industrial times (Hauglustaine and Brasseur 2001) to current peaks as high as 120 ppbv in North America. Ozone is a highly reactive compound capable of reacting with plant tissue, and the effects of elevated ozone on plant health have been investigated for nearly seven decades (Rich et al. 1964 and citations therein). Ozoneaffects plant health when it is taken up via stomata on leaves (Heath et al. 1994; Faoro et al. 2009). Once inside the plant tissue, ozone reacts to form a cascade of additional oxidizing agents, including oxygen radical, hydroxyl radical, or hydrogen peroxide (Heath 1994; Sandermann 2008; Fuhrer 2009). This cascade of oxidants can react with lipids and amino acids, alter hormone levels, and, at high concentrations, induce cell death (Heath 1994; Sandermann 2008; Fuhrer, 2009). Outward effects of this oxidation within the tissue include decreased plant growth, leaf stippling, flecking, bronzing, reddening, chlorosis, and even leaf senescence (Rich 1964; Heath 1980; Krupa and Manning 1988; Sanderman et al. 1998; Morgan et al. 2003; Ashmore 2005; Fiscus 2005; Feng et al. 2008).

Exposure to elevated levels of ozone can also impact flowers, and effects of elevated ozone on plants include flower senescence and a decrease in the production of flowers (Franzaring et al. 2000; Gimeno et al. 2004, Chappelka, A. H. 2002, Black et al. 2000) even at ambient ozone concentrations (Bergweiler and Manning, 1999). However, despite evidence that elevated ozone perniciously impacts flowers, there is a dearth of literature investigating if and how ozone impacts floral signals. As a result, it is unknown if ozone-induced changes to signals and rewards could alter pollinator preference of ozone-exposed plants and potentially impact pollination and thus both pollinator health and plant reproduction.

Ozone may alter floral visual and olfactory signal production and floral signals post-emission

Ozone could alter the production of floral signals either by damaging floral visual presentation or by altering the production of floral scents. Floral visual signals may be marred by exposure to ozone, as flowers, like leaves, can have stomata (Roddy et al. 2016; Slootweg et al. 1991) and may uptake ozone and experience the same oxidative stress as leaves, including chlorosis or flecking. If flowers express visual damage, it could impact their attractiveness to pollinators: flowers with florivore damage are less attractive to pollinators (Cunningham 1995; Lohman et al. 1996; Krupnick et al. 1999; McCall and Irwin 2006; Lucas-Barbosa et al. 2015; Sõber et al. 2010) indicating that visual damage to flowers could impact their attractiveness, although these studies do not control for other correlated traits, such as potential changes in floral scent in response to florivory.

Ozone could also cause changes in the production of floral scents that pollinators use to find and recall flowers. Numerous studies have found that whole plants exposed to ozone change their production of VOCs, with the majority of plant species experiencing an increase in their total emission of VOCs after exposure to ozone (Beauchamp et al. 2005; Llusia et al. 2002;

Vuorinen et al. 2004; Loreto et al. 2004; Khaling 2016; Li et al. 2015; Blande et al. 2007; Calfapietra et al. 2013; Heiden et al. 1999). This increase in plant VOC emission in response to oxidative stress may be driven by an increase in the plant's production of secondary metabolites, which are important chemicals in plant defense (Iriti and Faoro 2009). However, ozone-induced alterations in VOC production of some plants also include specific decreases in some monoterpenes that are common floral volatiles, including sabinene, linalool, and myrcene (Himanen et al. 2009; Pinto et al. 2007; Holopainen and Gershenzon 2010). While there is strong evidence that oxidative stress induced by ozone can alter volatile emission rates and blend compositions, no current studies have specifically examined the impact of ozone on floral and pollen volatiles, which are most likely to impact pollinator choices, nor have any current studies investigated if ozone-induced changes to the floral or pollen volatiles might impact pollinator choice.

In addition to inducing plant oxidative stress and causing plants to alter their production of floral signals, ozone can also alter floral scents post-emission. VOCs can be highly reactive with ozone (Atkinson and Arey 2003), and existing work finds that plumes of VOCs exposed to ozone experience both decreases in abundance of some compounds and a resulting compositional change (Li et al. 2016; Farre-Armengol et al. 2016).

Floral nectar and pollen quality and quantity may be altered by ozone exposure

Elevated tropospheric oxidants may also impact the production of floral rewards that are critical food-sources for pollinators and which incentivize and inform pollinator visitation (Weigman 2003; Knauer and Schiestl 2014). Studies find that both extrafloral (Blande et al. 2010) and floral (Witton 2016) nectar can increase with exposure to ozone. In addition, elevated ozone may alter nectar sugar allocation: one study finds a trend of decreasing sugar concentration with increasing

ozone concentration, although the response was not significant and may have been masked or driven by other variables in the field (Witton 2016). Another study on the broad bean *Vicia faba* finds that ozone exposure did not alter the nectar sugar concentration in the *V. fab*a nectar but did increase the amount of sucrose relative to other sugars in the nectar (Stabler 2016). Furthermore, ozone-induced changes to nectar composition of *V. faba* impacted pollinator perception. This difference in perception led to a difference in learning rate: European honeybees (*A. mellifera*) demonstrated a faster rate of learning but a shorter memory retention for odors associated with the ozone-exposed *V. faba* nectar type (Stabler 2016).

In addition to impacts on nectar, ozone-exposure may cause plants to produce less abundant or protein-rich pollen. Exposing pollen to ozone both in vivo (Schoene, Franz, & Masuch 2004; Black et al. 2000) and in vitro (Pasqualini et al. 2011; Feder, W. A. & Shrier, R. 1990) decreases the viability of the pollen, although the mechanism behind this decrease is unclear. Since a study on *Mimulus guttatus* found that inviable pollen had a significantly lower protein content and lower mass than viable pollen (Yeamans et al 2011), it is possible that ozone-exposure could cause pollen to be not only less viable but less abundant and nutritious. A direct study on the impacts of ozone on plants' pollen production finds that ozone-exposed plants had a reduced abundance of free amino acids in their pollen (Sabler 2016; Priberio et al. 2013), which may be reflective of an overall decrease in total pollen amino acid. Additionally, decreases in free amino acids may reduce a readily-available source of protein for insects that consume nectar or digest pollen by mixing it with sucrose (Erhardt & Baker 1990; Gilbert 1972; Eberhard et al. 2007) as free-amino acids in pollen are released into sucrose solutions such as nectar very quickly (Erhardt and Baker 1990; Gilbert 1980; Scogin 1986). Such ozone-induced changes to pollen abundance, free amino acid abundance, or protein content could negatively impact

pollinators that rely on pollen for protein and could also make plants proffering such poor rewards less enticing to foraging pollinators.

Specialist and generalist pollinator response to ozone-exposed plants

If ozone alters production of floral signals and rewards or alters floral scents post-emissions, then plant-pollinator interactions may be disrupted. However, pollinator response to ozone-exposed plants will vary not only with the ozone-induced changes to the plant but also with the foraging criteria and cue use of the pollinator. Specifically, specialist pollinators, which forage for pollen on just a few closely related taxa (Waser et al. 1986) and may use a distinctive subset of floral signals to locate their host plants (Schaffler et al. 2015; Brandt et al. 2015; Milet-Pinheiro et al. 2012; Burger et al. 2010), may respond differently to ozone-altered flowers of their host plants compared to generalist pollinators. Generalist pollinators can collect pollen from a range of plants from different families and can recognize a broad number of common floral signals (Bruce and Picketts. 2011). It is unclear if specialists' close relationship with host-plants will make them more or less likely to be deterred by ozone-induced changes in host plants: given their dependence on their few host plants, however, any disruption in attraction caused by ozone would most likely perniciously impact specialists.

Research Gap

There is a strong case to suggest that exposing flowers to ozone will affect the production of floral rewards and floral signals, both of which are critical to pollinator-plant interactions andto the continued success of the insects and plants involved. However, there is scant or nonexistent direct research on the impacts of ozone on floral signals and rewards or the impacts that such alterations may have on pollinator foraging choices. Furthermore, ozone may alter floral plumes

post-emission, but no current studies investigate how such plume alterations may impact a plume's attractiveness to a specialist pollinator. Here, we investigate the impact of short-term exposure to elevated ozone on floral production of signals and rewards, as well as the effects of ozone on floral olfactory signals post-emission. Finally, we assess how a specialist pollinator responds to plants exposed to ozone and plumes mixed with ozone post-emission.

RESEARCH QUESTIONS

(1) Does exposing plants to ozone result in changes in the production of floral signals or rewards in three different plant species?
(2) If there are changes in floral signals and rewards from ozone-exposed plants, will either a specialist or a generalist pollinator discriminate against ozone-exposed plants?
(3) Are post-emission floral signals altered by ozone, and if so, will a specialist pollinator discriminate against ozone-exposed plumes?

METHODS

Plant rearing

Cucurbita pepo var 'crookneck squash' and *N. alata* used for experiments assessing floral response to ozone exposure were raised in a greenhouse at the Max Planck Institute for Chemical Ecology in Jena, Germany. Petunia flowers of two varieties--'White cascade' and 'Multiflora' were purchased from both Lowes and Home Depot in Winchester, VA. Petunia plants were purchased at least 10 days prior to the start of the ozone-exposure experiment, and during the interim time, plants not exposed to treatments were housed in a greenhouse at Blandy

Experimental Farm, Boyce VA. *Cucurbita pepo* var 'crookneck squash' used for behavioral analysis and for floral volatile collections were reared from seed in Charlottesville, VA, with a 16hr light exposure. *Cucurbita pepo* plants were kept in a greenhouse at Blandy Experimental Farm immediately prior to experiments.

All three species of plants were exposed to either ozone-enriched air treatments or clean air treatments, and the effects of ozone exposure on flowers, including number of flowers and buds, size of flowers, pollen and nectar quantity and quality, and abundance and composition of floral and pollen scent were measured, although not all variables were measured for each flower species.

Petunia spp.

The effect of ozone on petunia plants has an unusually long history, with studies investigating the impacts of elevated ambient ozone on petunia growth, flower production, and overall health of different petunia varieties (Elkiey & Ormrod 1979a; Nouchi and Yanmazoe 1984). Some petunia varieties are marketed as 'ozone resistant' though the mechanism behind the observed resistance (Pitcher 1991) and the definition of this resistance is unclear. The two varieties used in this study, *Petunia* 'Multiflora' and 'White Cascade' were selected both because they were

available for purchase and because they have been found to be ozone susceptible in previous studies (Hanson et al. 1975; Elkiey, & Ormrod 1979a; Elkiey, & Ormrod 1979b).

Over the course of two summers at Blandy experimental Farm (May-August 2016 and 2017), the two varieties of petunia ('White Cascade' and 'Multiflora') were exposed to three ozone treatments: scrubbed clean air with ozone levels of ~12 ppbv, 80 ppbv and 120 ppbv. 'Multiflora' was also exposed to ozone at concentrations of 60 and 100 ppbv. 'Multiflora', which was tested in Summer 2016, was exposed to ozone for just 24 hours, while 'White Cascade' was exposed to ozone treatments for 72 hours in the Summer of 2017.

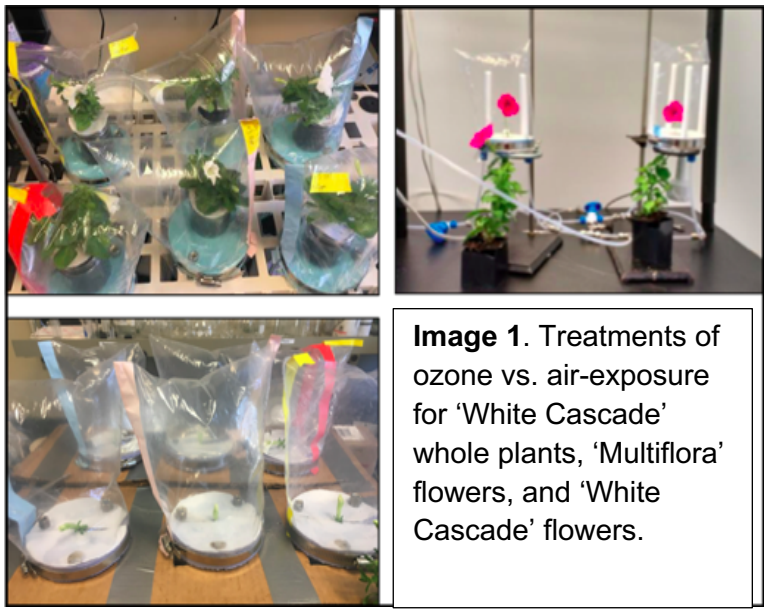


Image 1. Treatments of ozone vs. air-exposure for 'White Cascade' whole plants, 'Multiflora' flowers, and 'White Cascade' flowers.

In the Summer of 2016, petunia flowers were placed in individual chambers with 12.7cm diameter teflon bases and 13X17.78 cm telfon bags. Both chambers were then randomly assigned a treatment of either clean air (~12 ppbv Ozone) or to air enriched with either 60, 80, 100, or 120 ppbv ozone (Image 1A). The ozone absorption of the flowers was examined by measuring the concentration of ozone entering the chamber at 120 ppbv vs. leaving the chamber containing a flower.

In the Summer of 2017, isolated petunia buds and whole petunia plants of 'White Cascade' were exposed to the same three ozone levels (~10, 80, and 120 ppbv) for 72 hours before being assessed for impacts of ozone on floral rewards. Ozone for the 120 ppbv ozone treatment was generated using a Thermo Scientific Ozone Generator (Model 165 Thermo

Scientific in., Pittsburg PA) and ozone generated for the 80 ppbv treatment was generated with a Model 202, 2B technologies ozone calibrator. Ozone was measured using an ozone analyzer (Model 49i Thermo Scientific ozone analyzer).

The same ozone vs. air exposure chambers were used for 'White Cascade' whole plants and isolated petunia 'White Cascade' flowers in the Summer of 2017. Whole potted petunias were placed in one of six chambers made from13X17.78 cm teflon bags affixed with hose clamps to medical-grade round silicone-rubber bases, each with a diameter of 12.7X2.54cm thickness with a film of teflon on top. The bases were cut in half and held together with the hose clamps. Each silicone base with a teflon film rested on a plastic frame that held it in place (Image1B). 2l/min flows of either clean air or ozone-enriched air entered each chamber through an inlet in the silicone base, formed by a teflon tube that passed through the silicone base and was held in place with flush stainless steel nuts that connected to incoming or outgoing screw-inlets for airflow. The 1l flow of ozone-enriched or clean air exited the chambers via another identical outlet in the silicone base.

Flows going into each of the six chambers were controlled with Dweyer flow meters, so that 2l/min of air or ozone-enriched air entered each chamber. A positive pressure was built up in the chambers so that the air was pushed out of the chamber, through a flow meter, and into a manifold consisting of six solenoid valves. Flows of either air or ozone-enriched air from each chamber connected to one of the six solenoid valves which passed the flow either to an air scrubber, or to an ozone analyzer. The flow from each chamber was directed to the ozone analyzer for five minutes, with the manifold completing a rotation of chambers 1- 6 every 30 minutes. In the five minutes during which a chamber's flow was directed to the ozone analyzer, a data logger and logger net program recorded the ozone concentration of that chamber.

Data was first collected for whole petunia plants to ascertain if the petunias expressed symptoms of ozone-stress on their leaves, which has been previously described for petunias. When the damage to the leaves of the whole plants were observed, it was clear that ozone exposure time and concentration was sufficient to damage the petunia 'White Cascade' plant, and we further tested the effect of ozone on the flowers in isolation, removing leaves and their volatiles which may potentially protect plants and shield flowers by reacting with and quenching ozone near the plant.

Isolated flowers of petunia 'White Cascade' were then exposed to ozone using the same chambers and manifold used for whole-plant "White Cascade" ozone exposure. A stem of petunia with 1-2 buds was inserted into the base of the chambers via 2mm hole between the two halves of the silicone base--the two pieces of the base were then pushed together tightly with the hose clamp: because the silicone was of a rubbery texture, it created a fairly air-tight seal around the stem of the flower, and any extra space was plugged using silicone wax. A cardboard shelf was created to hold the silicone bases in place, with a space cut-out beneath each silicone base allowing the input and output of flows via teflon tubes (Image1C). Using the same experimental treatments as for the whole-petunia plants, isolated petunia buds were exposed to one of three ozone treatments (air, 80 ppbv, or 120 ppbv ozone) for three days. At the end of this time, most flowers had begun to bloom. Flowers that did not bloom were removed from the chambers but kept in the experiment room until they did bloom. If petunia flowers did not bloom within a day of being removed from the treatment chamber, they were removed from the study.

After ozone-exposure of whole plants of 'White Cascade" and isolated flowers of 'White Cascade' and 'Multiflora', the petunias were assessed for their production of nectar and pollen. Nectar was collected from each plant using 2ul microcapillaries (Drummond Scientific). Nectar sugar concentration was measured using a Extech portable sucrose analog refractometer. Due to

the very low nectar production for most petunias, all nectar samples were diluted with 1µl deionized water to provide enough volume to register on the refractometer. True concentration was then calculated proportionately. Pollen from each flower was collected using a tuning fork and the anthers further scraped with a spatula onto glass microscope slides. Samples were then weighed.

Effects of ozone on plant's pollen mass, nectar volume, and nectar sugar concentration were tested via regression, where variables were plotted against increasing ozone concentrations of ~12 ppbv ozone (control), 80 ppbv, and 120 ppbv ozone.

Because whole plants of 'White Cascade' were exposed to ozone, we further monitored 'White Cascade' for changes in number of buds, leaves, and flowers in exposed to either ~12ppbv ozone, 80ppv ozone, or 120ppbv ozone. Number of buds, leaves, and flowersof 'White Cascade' petunias for each treatment were then compared using a one-way ANOVA comparing the means of each group.

Additionally we compared floral plumes of 'White Cascade' after mixing with air and mixing with 120 ppbv ozone. This was achieved by placing three 'White Cascade' flowers in glass chamber with an airflow of 1l/min: the floral headspace then exited the bottle via a teflon tube and was split into a further two 2l bottles, where it entered at a rate of 0.5l/min apiece. In one 2l bottle, clean air was added at a rate of 0.5l/min, while in the other, air enriched with 120 ppbv ozone was added at the same rate of 0.5l/min. As the ozone-mixed or air-mixed floral headspace exited the two bottles, it passed into a 1'X1' teflon box with a stainless steel cap containing 5mm PDMS tubes. These PDMS tubes captured the floral headspace as it passed through the teflon box, and after 20 minutes, the PDMS tubes were removed and immediately put into a freezer, before being shipped in a cooler with ice packs to MPICE for further chemical analysis.

At MPICE, PDMS tubes with odors collected from air-exposed and ozone-exposed *N. alata* flowers and from ozone or air-mixed *N. alata* floral plumes were analyzed in individually using a thermo desorption unit (TDU, Gerstel, Germany) coupled to a temperature-programmable vaporizing unit (CIS 4, Gerstel, Germany), which was linked to an Agilent 7890A gas chromatograph (Agilent Technologies, CA) running in splitless mode and being connected to an Agilent 5975C mass spectrometer (electron impact mode, 70eV, ion source: 230◦C, quadrupole: 150°C,mass scan range: 33–350u). We used a nonpolar column (HP-5 MS UI, 30m length, 0.25mm ID, 0.25µm film thickness, J and W Scientific, USA) under constant helium flow of 1.1ml/min. The TDU temperature raised from 30°C to 200°C at a rate of 100°C/min and held for 5min. Volatized compounds were trapped within the CIS 4 cooled injection system at -50°C and subsequently injected into the GC. The GC oven was programmed to hold 40°C for 3min, to increase the temperature at 5°C/min to 200°C, then to increase temperature at 20°C/min to 260°C, which was kept for 15 min. Unprocessed data files were then exported and analyzed using the software package XCMS (Smith et al., 2006) implemented in R (R Core Team, 2014).

Nicotiana alata

Nicotiana alata is an herbaceous perennial in the Solanaceae family. *Nicotiana alata* is native to South America, ranging from southern Brazil, northeastern Argentina, and southern Paraguay (Goodspeed 1954). *Nicotiana alata* plants in this study were assigned to one of two treatments: 120 ppbv ozone or scrubbed clean air. Plants were exposed to ozone for 8 hours a day for four days, with scrubbed clean air flowing through all chambers the remaining 16 hours/day.

N. alata plants with at least 10 buds were placed in individual odor collection chambers with a ~20cm teflon bases, so that the stem of each plant was within the chamber, and the leaf

tissue and base were outside of the chamber (Image 2). Ozone concentrations were generated for each chamber by having a 3l/min flow of ozone-enriched air from a commercial ozone generator 'CleanPond ozone generator' enter through a one-way inlet at the top of each chamber. The concentration of the incoming ozone-enriched air was adjusted so that the air exiting each chamber by an outlet at the base was ~120 ppbv (throughout the experiment, mean ozone concentrations ranged between 112-131ppbv). Flows leaving the chamber were connected via a manifold to an ozone analyzer (Model 202 2B technologies). These valves completed rotation of chambers 1-8 every 40 minutes, so that each chamber was evaluated for its ozone concentration for a period of five minutes every 40 minutes.

Image 2. *N. alata* ozone vs. air-exposure chambers.

At the end of four days of eight-hour/day exposure to either scrubbed air treatment or 120 ppbv ozone treatment, *N. alata* plants were removed from their chambers and assessed for impacts of the ozone treatment on the number of flowers, size of flowers, nectar and pollen quantity and quality, and composition of floral volatiles.

Number of flowers and number of buds on each plant was counted. The size of each flower was assessed with three measurements: the tube length, the distance between the two most distant petal peaks, and the distance between the two most proximal petal peaks.

Nectar was collected via 50 µL calibrated glass micropipette tubes (Drummond "Microcaps" ; Drummond Scientific Co., Broomall, PA). Nectar sugar concentration was measured using an Extech portable sucrose analog refractometer. In addition to nectar sugar

concentration, we further assessed nectar sugar composition via LC-MS analysis. Nectar from flowers that had not been measured on the handheld refractometer were collected stored in 1ml vials for nectar sugar content analysis. The samples were then frozen, and later defrosted. A volume of 10 μL of nectar from each sample was then diluted with 0.5ml DI water. The mixture was vortexed and then analyzed in the LC-MS, along with blank DI water controls.

Pollen was also collected from ozone-exposed and air-exposed plants. Because pollen was frequently lost after it had burst from anthers, falling both inside and outside of the flower where it could not be easily discerned from other flowers' pollen or retrieved, pollen was collected by removing anthers before they had burst open. Anthers were then dissected: underdeveloped pollen was easy to assess and remove from the study (Image 3). For pollen sacs with developed pollen, the pollen was removed by slitting open the pollen sac and tapping out the contents. Residual pollen was gently swiped off with a pair of fine-tipped tweezers. Pollen was then dried for 12 hours at 50C, and weighed.

Image 3. *N. alata* pollen collection

Number of flowers, number of buds, tube length, distance between petals, amount of nectar, nectar sugar concentration, amount of glucose, sucrose, and fructose in nectar, and the amount of pollen were compared between ozone-exposed and air-exposed *N. alata* flowers using either t-tests or Wilcoxon signed rank test, depending on the data's normality after attempted transformations (Fig 4).

To assess whether ozone-exposure altered the production of floral volatiles, the floral headspace of two *N. alata* flowers per *N. alata* plant was collected at the end of the ozone or air exposure treatments. Two flowers that had been exposed either to elevated ozone or to air were

placed in a small airtight box with a 0.5l/min inflow of scrubbed clean air. The air was pushed through the box with the flowers, and ran via a teflon tube into another small box made of teflon with a screw-tight lid which contained five PDMS tubes. Volatiles were collected onto the PDMS tubes in this manner for twenty minutes. PDMS tubes with the volatiles from the ozone-exposed vs. air exposed *N. alata* flowers were then run through the GC-MS following the same procedure described for petunia flowers.

N. alata compounds were identified as present in the ozone-exposed flowers but not in the air-exposed flowers only if they were found in at least 50% of ozone-exposed plants and none of the air-exposed plants, and vice-versa. The total volume of volatiles from ozone-exposed and air-exposed flowers was log-transformed and then compared using a t-test after removing the siloxane compounds that originate from the PDMS tubes.

I further examined how *N. alata* volatiles were altered by ozone post-emission. As with petunias, two flowers of *N. alata* still attached to the plant were placed in a glass odor collection chamber. An airflow of 1l/min entered the glass odor collection chamber and carried floral headspace out of the chamber via a teflon tube and was split into a further two 2l bottles, where it entered at a rate of 0.5l/min apiece. In one 2l bottle, clean air was added at a rate of 0.5l/min, while in the other, air enriched with 120 ppbv ozone was added at the same rate of 0.5l/min. As the ozone-mixed or air-mixed floral headspace exited the two bottles, it passed into a ~2.54cm square teflon box with a stainless steel cap containing 5mm PDMS tubes. These PDMS tubes captured the floral headspace as it passed, and after 20 minutes, the PDMS tubes were removed and either stored in a -20C freezer or ran immediately through a GC-MS. The GC-MS procedure was the same used for analyzing petunia volatiles.

Peak area values for the chemical scent traces generatedby the GC-MS were log transformed to ensure normality, and finally compared by a principal component analysis.

Cucurbita pepo

Cucurbita pepo is a variety of crookneck or yellow squash in the family Cucurbitaceae. It is a bushy monecious summer squash that originates in the eastern United States, and was domesticated as early as 10,000 years ago (Smith 1997). *Cucurbita pepo* plants were placed in individual glass odor collection chambers and exposed to either a scrubbed air treatment or a 120 ppbv ozone-enriched air treatment for eight hours a day for three days. At the end of this time, there was visible damage to *C. pepo* leaf tissue (Image 5B). *C. pepo* plants were then removed from the chambers and their floral traits and floral rewards were assessed.

Ozone and scrubbed air treatments

Cucurbita pepo plants were selected for the experiment if they had at least two buds that were likely to bloom after three days of treatment. Once *C. pepo* plants with appropriately-aged buds had been selected, the potted plants were randomly assigned to one of two 6l glass odor collection chambers with teflon bases (image 5A). A hole in the telfon base intended for plant stems was plugged with silicone wax. These glass treatment chambers were located within a laboratory, and had grow-lights hung above the chambers, which were activated between 7AM-9PM daily. Either clean air or 120 ppbv ozone-enriched air entered these chambers via an inlet at the top of each chamber at a rate of 4l/min. The flow of either air or ozone-enriched air was then pulled from the chambers at a rate of 3.5l/min from an outlet at the bottom side of each chamber via pumps. As the flow of ozone-enriched air exited the chamber, it was directed into an ozone analyzer (Model 202 2B technologies Ozone analyzer). *Cucurbita pepo* plants in the ozone treatment were exposed to ozone for 8 hours a day (~10AM-6PM), for three days, with scrubbed air being substituted for the ozone-enriched air for the remaining 16 hours per day.

After three days of 120 ppbv ozone or scrubbed air treatment, *C. pepo* plants were removed from their individual chambers and assessed for differences in number of buds, nectar and pollen amount, nectar and pollen quality, and whole plant, floral, and pollen volatiles.

Pollen was collected at two time intervals: immediately after the third day of ozone-exposure, and then five days after plants had been removed from their ozonation or air-exposed chambers. Pollen was collected using a tuning fork to buzz pollen onto glass microscope plates. Residual pollen was gently swiped off with a pair of fine-tipped tweezers. Pollen was dried in an oven for 12 hours at 50°C, then weighed so that the weight of pollen from ozone-exposed and air-exposed *C. pepo* plants could be compared. In addition, pollen was further analyzed for its Carbon: Nitrogen (C:N) ratio using an elemental analyzer. Pollen was collected from 3-4 flowers per *C. pepo* plant for 18 plants. Pollen was dried at 50°C for 36 hours, and stored in -20°C freezer before again being dried. Dried pollen samples from a single plant were then homogenized using a Wig-L-bug, and tinned together to make 6mg samples for the elemental analyzer, which analyzed the C:N content of the pollen.

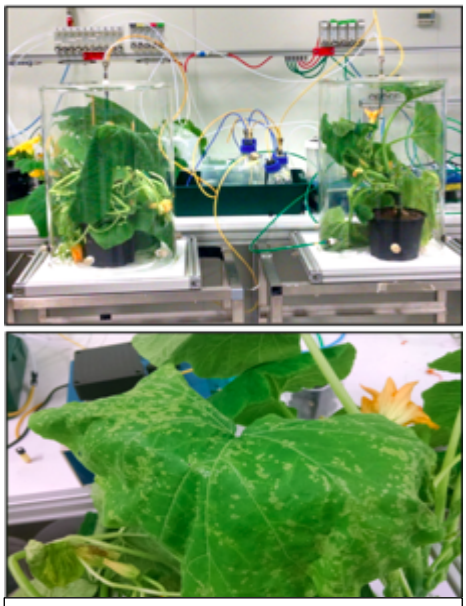


Image 4. *C. pepo* ozone vs. air-exposure chambers, and ozone damage to leaves.

Nectar was collected via 50 L calibrated glass micropipette tubes (Drummond "Microcaps" ; Drummond Scientific Co., Broomall, PA). Nectar sugar concentration was measured using an Extech portable sucrose analog refractometer.

In addition to nectar sugar concentration, we further assessed nectar sugar composition via LC-MS analysis. Nectar from flowers that had not been measured on the handheld

refractometer was stored in 1ml vials for nectar sugar content analysis. The samples were then frozen, and later defrosted. A volume of 10 µL of nectar from each sample was then diluted with 0.5ml DI water. The mixture was vortexed and then analyzed in the LC-MS, along with blank DI water controls.

Amount of nectar, nectar sugar concentration, amount of glucose, sucrose, and fructose in nectar, and the amount of pollen were compared between ozone-exposed and air-exposed *C. pepo* flowers using either t-tests or Wilcoxon signed rank test, depending on the data's normality after attempted transformations. C:N content of pollen for air exposed and ozone-exposed *C. pepo* flowers both immediately after treatment and then again after five days of treatment was compared using an ANOVA(Fig 4).

Ozone's impact on volatile production was assessed both for the whole *C. pepo* plant, and for *C. pepo* flowers and for pollen separately. To collect whole plant volatiles, clean air was run through the treatment chambers housing the *C. pepo* plants at a rate of 1l/min. Headspace was pulled from the chamber with a vacuum pump set to 0.5l/min, and passed over the PDMS tubes for 20 minutes.

To collect isolated floral volatiles from air-exposed and ozone-exposed *C. pepo* flowers, individual flowers were placed in 1l glass collection chambers with a teflon base that enclosed the stem, so that only the flower and part of the stem were in the chamber, with the rest of the plant being held outside of the chamber. Any gaps between the stem and the teflon base were plugged using silicone wax. Scrubbed air flowed into the chamber via an inlet at the top at a rate of 0.5l/min, and the floral volatile headspace was pulled from the chamber via an outlet connected with teflon tubing to a pump pulling at 0.5l/min. Between the chamber and pump was a screw-tight teflon box with a stainless steel lid which contained five PDMS tubes. Floral headspace was passed over the PDMS tubes for 20 minutes.

Cucurbita pepo pollen odor was collected by combining the pollen of three ozone-exposed or air-exposed plants, and putting the pollen in two (50ml) glass bottles. Each bottle was sealed, and placed on a hot-plate set to 30ºC. After an hour on the hot plate, a red SPME fiber (Supelco, 100um polydimethylsiloxane fiber) was inserted into the glass bottle via a tube connector port: the fiber extended into the bottle just above the pollen, and the base of the SPME fiber covered the port. Odors were collected in this manner for 20 minutes.

Following the scent collection for whole *C. pepo* plants, *C. pepo* flowers, and *C. pepo* pollen, PDMS tubes were analyzed individually using a thermo desorption unit (TDU, Gerstel, Germany) coupled to a temperature-programmable vaporizing unit (CIS 4, Gerstel, Germany), which was linked to an Agilent 7890A gas chromatograph (Agilent Technologies, CA) running in splitless mode and being connected to an Agilent 5975C mass spectrometer (electron impact mode, 70eV, ion source: 230◦C, quadrupole: 150°C,mass scan range: 33–350u). We used a nonpolar column (HP-5 MS UI, 30m length, 0.25mm ID, 0.25µm film thickness, J and W Scientific, USA) under constant helium flow of 1.1ml/min. The TDU temperature raised from 30°C to 200°C at a rate of 100°C/min and held for 5min. Volatized compounds were trapped within the CIS 4 cooled injection system at -50°C and subsequently injected into the GC. The GC oven was programmed to hold 40°C for 3min, to increase the temperature at 5°C/min to 200°C, then to increase temperature at 20°C/min to 260°C, which was kept for 15 min. Unprocessed data files were then exported and analyzed using the software package XCMS (Smith et al., 2006) implemented in R (R Core Team, 2014).

Peak area values were log transformed to ensure normality and finally compared by a principal component analysis.

Cucurbita pepo compounds from whole plants, flowers, and pollen were identified as present in the ozone-exposed pollen but not in the air-exposed pollen only if they were found in

at least 50% of ozone-exposed plants and none of the air-exposed plants, and vice-versa. The total volume of volatiles from ozone-exposed and air-exposed flowers was log-transformed and then compared using a t-test, after removing siloxane compounds that originate from the PDMS tubes.

Finally, we examined whether post-emission *C. pepo* plumes are altered by exposure to ozone: This was achieved by placing four *C. pepo* flowers in the 1l glass odor collection chamber, still attached to the plant. Clean air entered the chamber at a rate of 0.5l/min, and was pushed out of the chamber into a teflon tube that was split via a stainless steel y-connector. One half of the floral headspace was then passed through a flow-meter set to 0.25l/min, and into a 2l bottle via a one-way flow connector; the floral headspace was then joined by 0.25l/min of 120 ppbv ozone generated by a Model 202, 2B technologies ozone calibrator. The other half of the floral headspace was also directed into a 2l bottle, where it mixed with 0.25l/min of scrubbed clean air. Floral headspace exiting either mixing bottle was passed through a 1X1" teflon box with a screw-tight stainless steel lid containing 5mm PDMS tubes. Ozone-exposed and air-exposed plumes were collected on these PDMS tubes for 20 minutes. The PDMS tubes were then analyzed with the same GC-MS setup described above for floral, whole plant, and pollen scent from *C. pepo* flowers exposed to either ozone or to air.

Peak area values obtained from the GC-MS analysiswere log transformed to ensure normality and finally compared by a principal component analysis. Individual peaks that dominated PCA analysis were further selected for t-test or Wilcoxon test comparisons.

Insect behavior assays

I next assessed whether exposing plants to ozone impacted their attractiveness to pollinators. I tested the response of a generalist (*Apis mellifera*) and a specialist (*Peponapis pruinosa*) bee to

ozone-exposed and air-exposed squash plants presented in a plywood foraging chamber. As a Cucurbita specialist, *P. pruinosa* forages exclusively on pollen from cucurbits, and females nest at the base of Cucurbit plants. In contrast, *Apis mellifera* is a broad generalist foraging on plants from many different families; however, it has been documented as one of the major pollinators visiting squash crops (Julier and Roulston 2009). Squash bees for this experiment were collected at Blandy Experimental Farm community garden from flowering Cucurbita plants, and European honey bees were collected while entering or exiting a feral *A. mellifera* colony at Blandy Experimental farm.

Plant preparation for behavior assays

Cucurbita pepo used for pollinator choice experiments were raised from seed in a greenhouse at Blandy Experimental Farm, Boyce VA. Plants had an augmented light period of 16 hours. When *C. pepo* plants began developing ~10-day old buds, they were randomly assigned to one of eight glass chambers, where they would be exposed to either a 120 ppbv ozone or air treatment. Glass chambers were not sealed at the bottom, but rested on the soil within the *C. pepo* pots. Ozone and air treatments consisted of individual glass jar chambers (1 gallon) chambers with a steady 2l/min inflow of either scrubbed clean air or ozone-enriched air for a 12-hour period every 24 hours for six days. Two grow lights were placed above the *C. pepo* chambers in the lab, and plants were exposed to light throughout their time in the treatment chambers. Rotating plants in and out of ozone or air-exposure chambers was necessary as the glass chambers had an adverse effect on plant health after an extended period of continuous time, as ascertained by the advent of molding and bud-loss in preliminary air-exposed *C. pepo* trials.

After a total of 72 hours of exposure to either ozone-enriched air or scrubbed clean air over the course of six days, *C. pepo* plants in ozone-exposure chambers showed visible oxidative

damage. Leaves of ozone-exposed *C. pepo* plants were heavily mottled with yellow and brown spots. At this point, plants were removed from their chambers and used for bee choice assays. After bee choice assays were concluded for a given day, the number of buds and flowers on each plant was counted, and the chlorophyll content of the leaves was measured using a chlorophyll measurer (Model MC-100 Apogee instruments).

Insect collection and preparation

Peponapis pruinosa were collected from Blandy Experimental Farm community garden, Boyce VA, or from WaterPenny Farm, Sperryville, VA. Once collected, the bees were kept for at least one day (24hrs) in a mesh cage with a *C. pepo* plant and artificial paper flowers containing 1ml of 40% sugar water. Insect mortality for one day was very low, but increased over time: to improve bee longevity, I hand fed squash bees sugar water daily, removing them from the mesh cage and touching their proboscis with a Q-tip soaked in sugar water. When the sugar water touched the bees' proboscis, they typically fed eagerly for 30 seconds or more. After several days in the cage, some *P. pruinosa* could be observed feeding on artificial flowers, but were still hand-fed. *Apis mellifera* were collected as they entered and exited a wild colony at Blandy Experimental Farm. Honey bees were also kept for 24 hours in a mesh cage containing *C. pep*o and artificial flowers with 40% sugar water: unlike the *P. pruinosa*, the *A. mellifera* readily fed at the artificial flowers and did not need to be hand-fed.

Behavior assays

Response to ozone-exposed *C. pepo* plants

Behavior assays were conducted in a 2X4’ plywood box with a bridal veil top held in place with binder clips to allow easy removal of the veil. On one side of the box, there was a small

(~2.5X2.5”) square entrance cutout where individual bees were released into the chamber. Once bees had entered the chamber, this entrance was covered with a plastic patch fastened into place with velcro. At the far end of the box were placed two potted *C. pepo* plants, one which had been exposed to ozone, and one which had been exposed only to scrubbed air. A sony handycam video camera was set up on a tripod above the behavior box. The video captured both potted plants and a dowel stuck inside each pot where the plant ID was written on a tag and clearly visible (Diagram 1). Between the hours of 5:30am-10:30am, individual female *P. pruinosa* or *A. meliferra* were transported from their respective mesh cages to the plywood box via falcon tubes. Falcon tubes were tilted at an angle, encouraging the bees to crawl to the top of the tube, which rested at the entrance of the behavior box. As bees approached the lip of the vial, the vial was jostled slightly, encouraging the bees to fly into the chamber. Bees were then given five minutes to fly in the box. The bees first and subsequent approaches to either the ozone-exposed or the air-exposed *C. pepo* plant were recorded, and the first landing was also recorded and timed (approaches to the flowers that did not result in landings were too quick to accurately measure using a stopwatch). The great majority of bees that landed on a flower did not leave the flower during the five minutes of filming, so first landing was taken to be the best representation of pollinator choice in this experiment. Bee preference for ozone-exposed vs. air-exposed squash plants was assessed with a negative binomial analysis in RStudio. Negative binomial analysis was chosen rather than a simple chi-square test because it could account forthe additional variable of pairs of ozone and air-exposed plants presented to the bees in the foraging chamber. Ozone and air-exposed plant pairs which were treated as a fixed effect that could block for bee response, as the air and ozone-exposed plant pairs were switched after every 2-5 bees. The negative binomial analysis further included the bee choice and plant pair interaction effect.

Response to ozone-exposed *C. pepo* plumes

In addition to testing squash bee's response to ozone-exposed *C. pepo* plants, I further tested their response to *C. pepo* floral plumes that had been mixed with air, or those that had been mixed with 120 ppbv ozone.

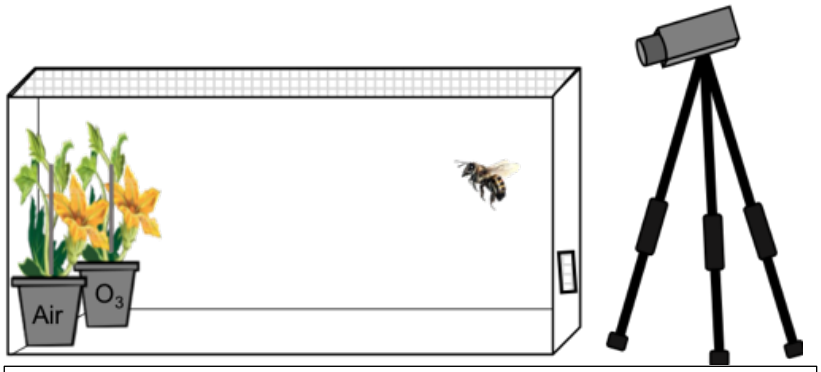


Diagram 1. *Foraging chamber for P. pruinosa choice tests testing preference for air vs. ozone-exposed C. pepo flowers.*

Squash bees that had been in a mesh cage containing squash flowers for at least 24 hours prior to experiments were put into individual falcon tubes and transported to the same behavior box. For this experiment, however, no *C. pepo* flowers were located at the far side of the box. Instead, squash bees were given the choice between two artificial flowers, one emitting the headspace of four *C. pepo* flowers mixed with scrubbed clean air or 120 ppbv ozone, and the other emitting scrubbed clean air.

The two artificial flowers were made by layering two discs of orange foam of different shades (internal disk diameter of 1", external disc diameter 3.5"). These flowers were affixed to the far side of the behavior chamber by a 5mm teflon tube, which passed through the center of the artificial flowers and also passed through holes drilled into the plywood box. The flowers were held steady by supporting the teflon tubing with silicone wax. Through this teflon tube, either clean air or floral headspace, exposed either to air or ozone, entered the behavior chamber. Ozone-exposed and air-exposed floral plumes of *C. pepo* flowers were produced in the following manner: a *C. pepo* plant with at least four flowers was placed in a 2l airtight glass chamber with

three ports, manufactured by Analytical Research Systems (Gainsville FL). Scrubbed clean air flowed through this chamber at a rate of ~1l/min and passed through a flow meter at a rate of 0.25l/min into a 1l glass mixing bottle. In the glass mixing bottle, 0.25l of either ozone-enriched air or scrubbed clean air also entered the glass bottle via a stainless steel one-way y-connector. As the ozone-exposed or air-exposed floral plume exited the bottle, it moved via a teflon tube first through a flow meter set at 0.25l/min and then connected to the artificial flower via a hole drilled in the back of the behavior chamber. Thus, floral headspace mixed with either ozone or air was emitted from one artificial flower, while the other artificial flower was connected to the same clean air source, which passed through a flow meter and directly into the behavior chamber from the other artificial flower.

RESULTS

Petunia spp.

Effects of ozonation on petunia rewards

Both varieties of petunia exposed to ozone showed some different but overlapping responses in production of rewards under increasing ozone treatments. Petunia nectar volume increased with increasing ozone levels for all three species, although the slope was only significantly above zero for petunia whole plant ozone-exposed 'White Cascade' and for isolated flowers of 'Multiflora.' After removing flowers that did not produce nectar, both "White Cascade" whole plants exposed to ozone and 'Multiflora' flowers exposed to ozone had a trend of increasing nectar volume with increasing ozonation. Ozone did not seem to affect the petunia 'White Cascade' more

dramatically when only the single flower was exposed rather than when the whole plant was exposed to ozone.

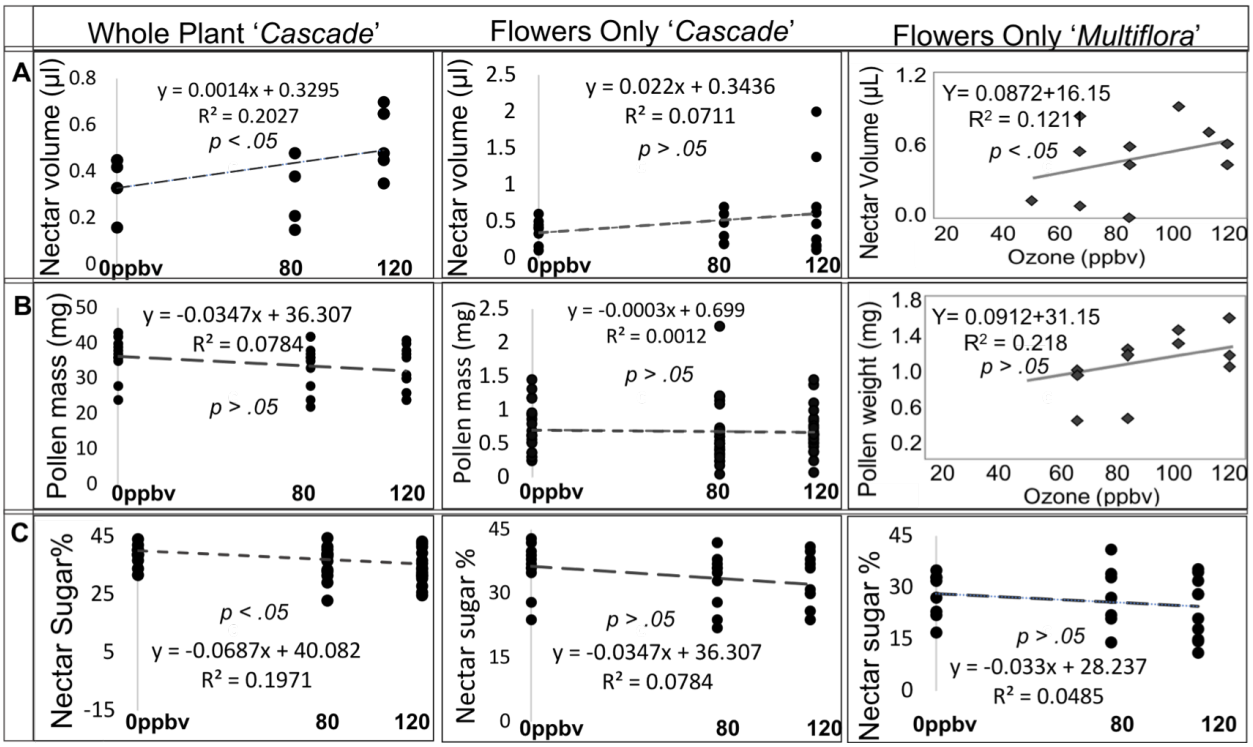


Fig 1. Effects on pollen and nectar for Petunia flowers with and without whole plant exposure to ozone. **A**. Ozone increased nectar volume in Whole plant 'White Cascade' (linear regression: p-value= 0.049) and flowers of 'Multiflora' (linear regression: p-value = 0.032) **B**. Pollen mass significantly increased with ozone exposure in petunia 'Multiflora' (linear regression: p-value = 0.0241), but did not show the same trend in other treatments **C.** Nectar sugar concentration showed a decline in response to increasing ozone concentration for petunia 'White Cascade' flowers in isolation (linear regression: p-value 0.0446).

Effects of ozonation on petunia flowers, and numbers of buds, leaves and flowers

Ozonation caused ‘White Cascade’ petunia plants to decrease the size of their flowers, although it did not affect tube length. Whole plants of “White Cascade” exposed to ozone also showed a decline in the number of buds and leaves but not flowers when compared to ‘White Cascade’ plants exposed to clean air for the same amount of time. Ozone concentration was measured before and after leaving the chamber to determine ozone adsorbption and absorption, although the observed decline in ozone concentration may have been due to either absorption of ozone by the flower or by quenching of ozone from humidity or VOCs released from the flower.

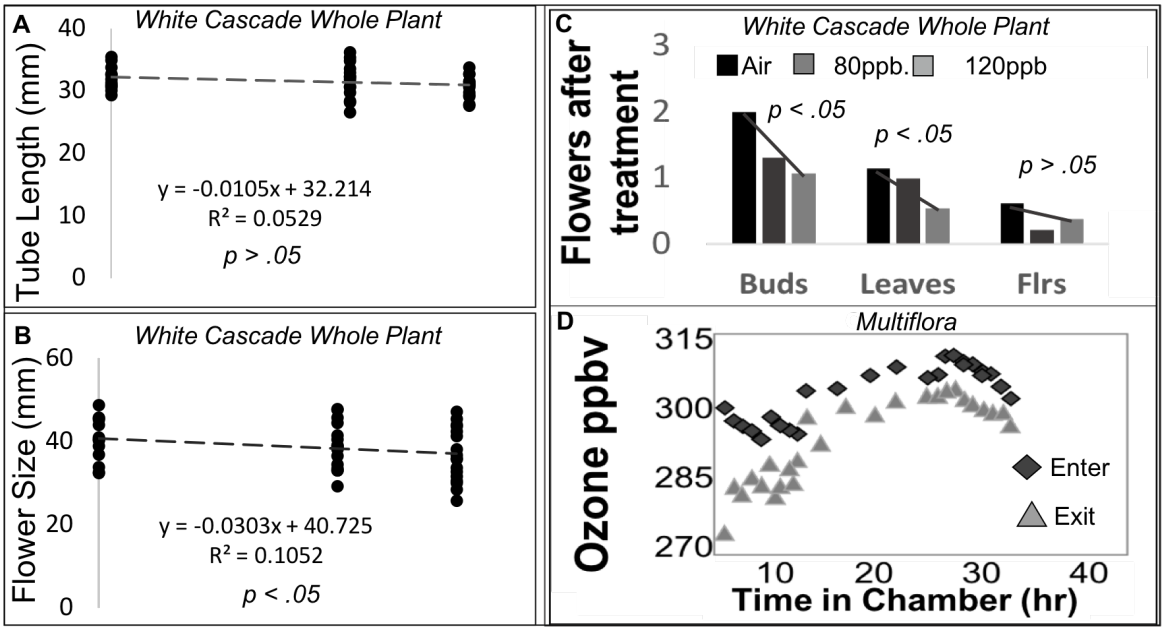


Fig 2 Petunia whole plants exposed to ozone **A.** Tube length was not affected by ozone exposure (y = -0.0105x+32.214, p-value = 0.641) **B.** Flower size decreased with increasing ozone concentrations (y = -0.0303x+40.725, p-value = 0.459). **C.** ‘White cascade’ plants exposed to ozone showed a fewer buds (ANOVA: F= 3.986, p-value = 0.0152) and leaves (ANOVA: F= 4.114, p-value = 0.0289) but not flowers (ANOVA: F= 0.783, p-value = 0.465) after exposure to ozone**. D.** Sample traces showing a time lapse of the ozone concentration entering and exiting a chamber containing one petunia ‘Multiflora’ flower.

Impacts of ozone on petunia "White Cascade' plumes post-emission

Ozone mixing with the post-emission plume of petunia caused a reduction in abundance of some compounds as assessed visually by sample traces and separation in principal component analysis.

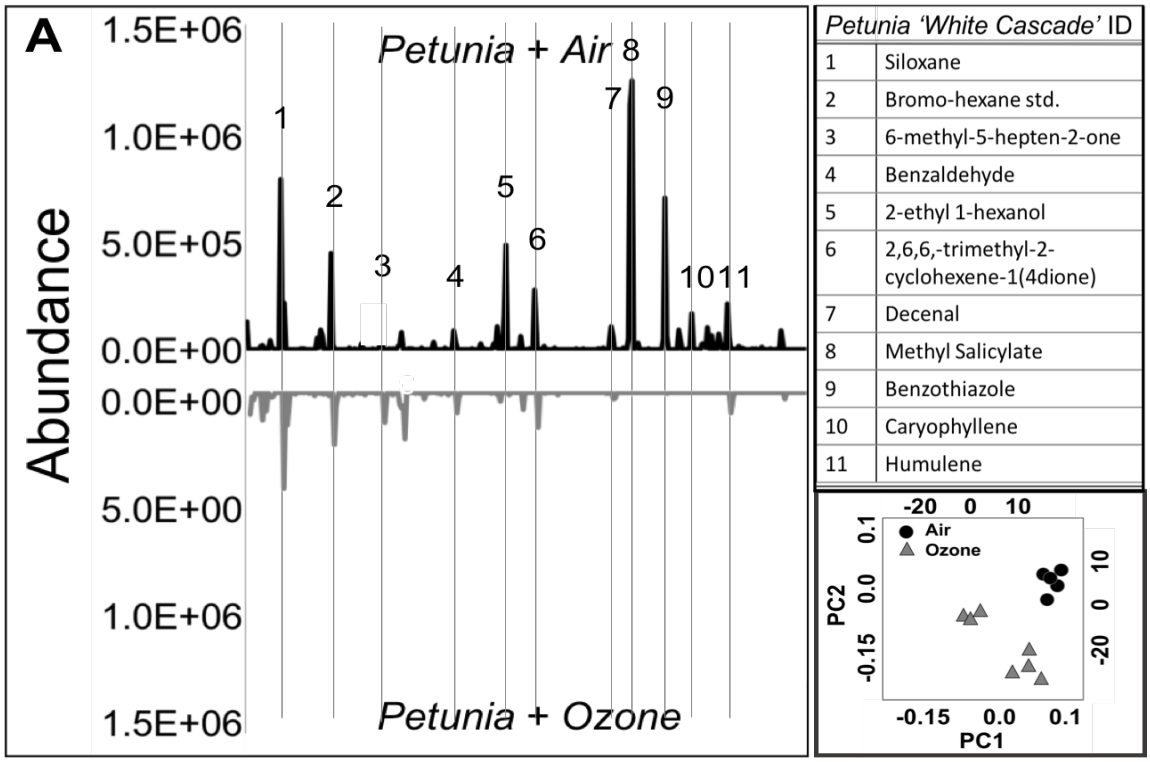


Fig. 3. Petunia floral plumes exposed to ozone. **A.** Sample trace of a single petunia plume mixed with air (black) and mixed with ozone (grey).**B**. Numbered peaks are identified by the NIST library (R-match > 90%). **C.** Plumes exposed to ozone vs. air create visually distinct groups in PCA analysis of seven petunia flowers.

Nicotiana alata

Exposure to ozone affected both floral rewards and floral signals of *N. alata*. Ozone exposure caused a decrease in the number of flowers, number of buds, and flower size of *N. alata.*

Furthermore, while ozone exposure did not affect pollen volume or pollen weight, there was a non-significant trend decreasing nectar sugar concentration as analyzed by LC-MS, potentially driven by significant decreases in fructose in ozone-exposed flowers.

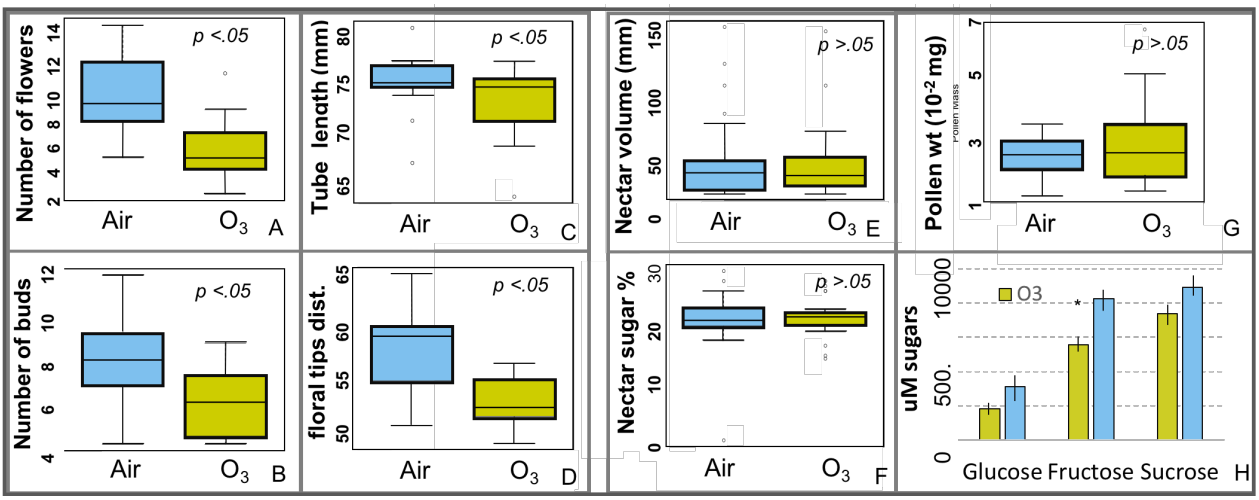


Fig. 4. Effects of ozone on flower size, abundance, and rewards. *N. alata* exposed to ozone have significantly fewer **A.** flowers (t-test: t=5.1, p-value = 1.08e-5) and **B.** buds (t-test: t=3.27, p-value =0.00233). Ozone-exposed *N. alata* flowers were also smaller in size, with **(C)** shorter tube length (Wilcoxon:W=287, p-value = 0.04021) and **(D)** shorter distances between the tips of the two most-distant petals (t-test: t = 2.9086, p-value = 0.00737). Ozone exposure did not significantly affect **(E)** nectar volume (Wilcoxon: W= 594, p-value =0.881), **(F)** nectar sugar concentration (Wilcoxon: W=594, p-value =0.851), or **(G)** pollen mass (Wilcoxon: W= 218.8, p-value = 0.8347). **(H)** Ozone exposure did not cause a significant change in the µM abundance of either glucose (t-test: t=1.13, p-value = 0.314), or sucrose (t-test: t=1.09, p-value = 0.412), but did significantly affect the abundance of fructose (t-test: t=2.81, p-value = 0.0091).

Visual damage to ozone

Image 5. Visual representation of ozone's impacts on *N. alata* flower size and visual presentation, and impacts of elevated ozone on floral scent production. In addition to decreasing flower size **(A)** Ozone-exposure caused browning of floral sepals. Ozone-exposed sample shown in **(B)** and air-exposed sample shown in **(C)**

Plumes of N. alata from air and ozone-exposed plants, and plumes mixed with ozone and air post-emission.

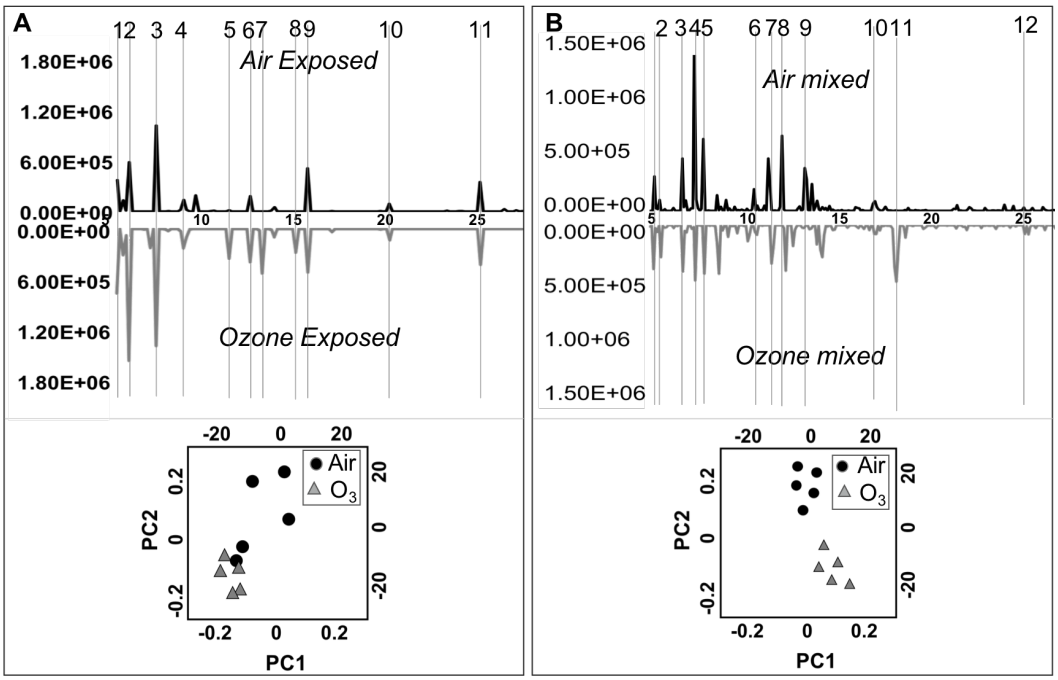


Fig. 5. *N. alata* floral plumes from ozone vs. air-exposed plants, and floral plumes of *N. alata* mixed with ozone vs. air. **A.** Sample trace of plumes from air-exposed *N. alata* (black) and ozone-exposed *N. alata* (grey). The total amount of volatiles from plants exposed to ozone increased (log transformed t-test: t=2.118, p-value = 0.04969). PCA showing the divergence in

the plumes of five ozone and air exposed *N. alata* flowers. **B.** Sample trace of *N. alata* mixed with air (black) and mixed with ozone (grey). PCA showing the divergence of five ozone and air-mixed *N. alata* plumes.

Table 2
N. alata volatile ID

N. alata O_3 Exposed ID		*N. alata* O_3 Mixed ID	
1	Syn-3-methyl-butyl-aldoxime	1	Syn-3-methyl-butyl-aldoxime
2	Anti-3-methyl-butyl-aldoxime	2	Anti-3-methyl-butyl-aldoxime
3	Bromo-Hexane std.	3	Bromo-Hexane std.
4	Beta-myrcene	4	Heptanal
5	Methyl Salicylate	5	6-methyl-5-hepten-2-one
6	Decanal	6	Beta-myrcene
7	D-Limonene	7	Decanal
8	Beta-Caryophyllene	8	D-Limonene
9	Eucalyptol	9	Eucalyptol
10	Linalool	10	Linalool
11	Trans-Nerolidol	11	Nonanal
		12	Trans-Nerolidol

Table 2. Identification of floral volatiles from ozone-exposed and air-exposed *N. alata* plants and from *N. alata* plumes mixed with either are or ozone. Numbered peaks are identified by the NIST library (R-match > 90%).

Cucurbita pepo

Impacts of elevated ozone on C. pepo *rewards*

Elevated ozone exposure increased nectar volume, but did not significantly impact nectar sugar concentration, nectar sugar composition, or pollen mass, although there was a significant effect of ozone on pollen C:N ratio after a delay of five or more days post-ozone exposure.

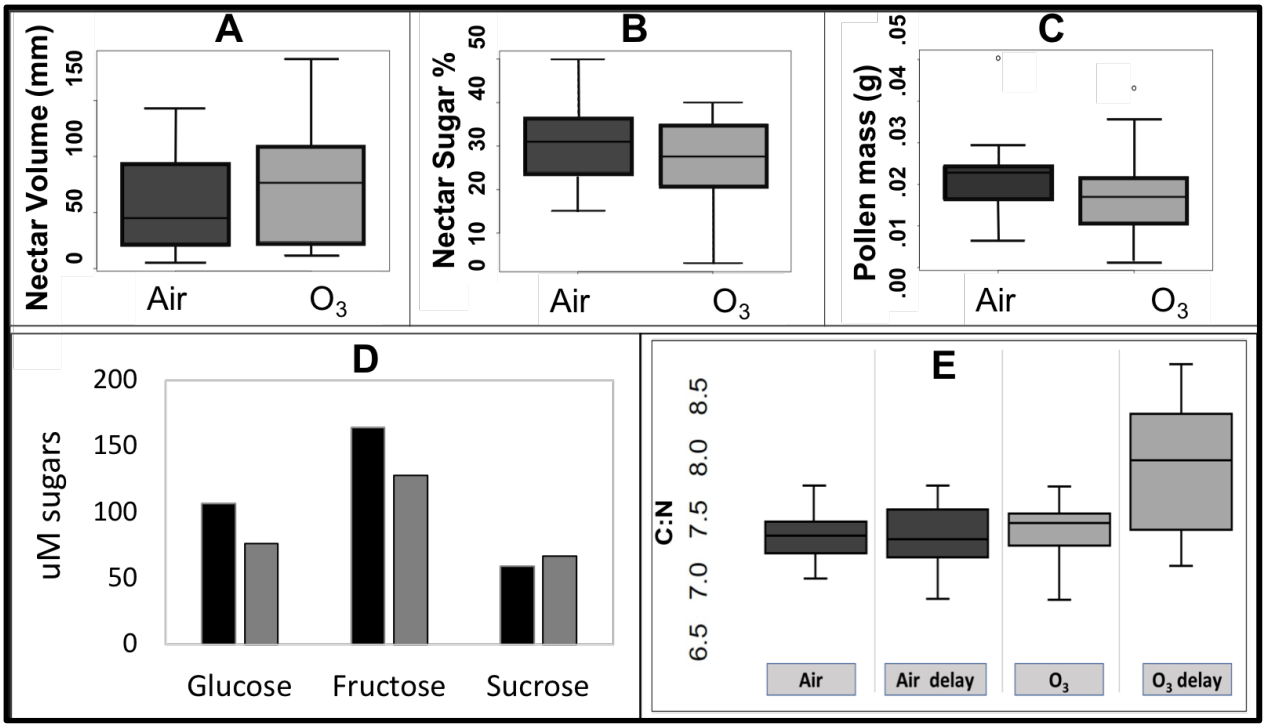


Fig. 6. Effects of pollen and nectar for *C. pepo* exposed to ozone. **A.** Exposure to 120 ppbv ozone caused a significant increase in *C. pepo* nectar volume (Wilcoxon V= 26.5, p-value =0.034), but had no significant effect on **B.** nectar sugar concentration (Wilcoxon V= 541, p-value=0.05367), **C**. or pollen mass (Wilcoxon V= 191, p-value =0.252) **D.** Ozone did not significantly alter nectar sugar composition, causing non-significant decreases in glucose (t-test: t=1.12, p-value = 0.364), or sucrose (t-test: t=1.38, p-value = 0.412), or fructose (t=1.81, p-value = 0.091) **E.** Ozone exposure caused an increase in pollen C:N after a five day or more delay (ANOVA: F= 4.46, p-value = 0.02201).

Impacts of ozone-exposure on plume production for C. pepo *whole plants,* C. pepo *flowers, and* C. pepo *pollen*

Ozone altered the production of plumes of *C. pepo* whole plants, flowers, and pollen: ozone exposure to *C. pepo* plants increased the total amount of volatiles produced by *C. pepo* whole

plants, but did not increase the total volume of volatiles produced from isolated *C. pepo* flowers or pollen. *C. pepo* flowers however did show a strong increase in the compound 1,4-dimethoxybenzene (compound#6) (t-stat: 1.943, p-value = 0.00365). Furthermore, the increase in1,4,-dimethoxybenzene was largely responsible for the separation in PCA groupings: 1,4-dimethoxybenzene had the largest eigenvectorwhich in turn had the largest eigenvalue (eigenvalue proportion = 0.571856). There was no evidence that changes in pollen volatiles from ozone-exposed *C. pepo* plants were similarly driven by just one key compound, although the pollen odors from ozone-exposed and air-exposed *C. pepo* plantsformed visually separate groupings in PCA analysis.

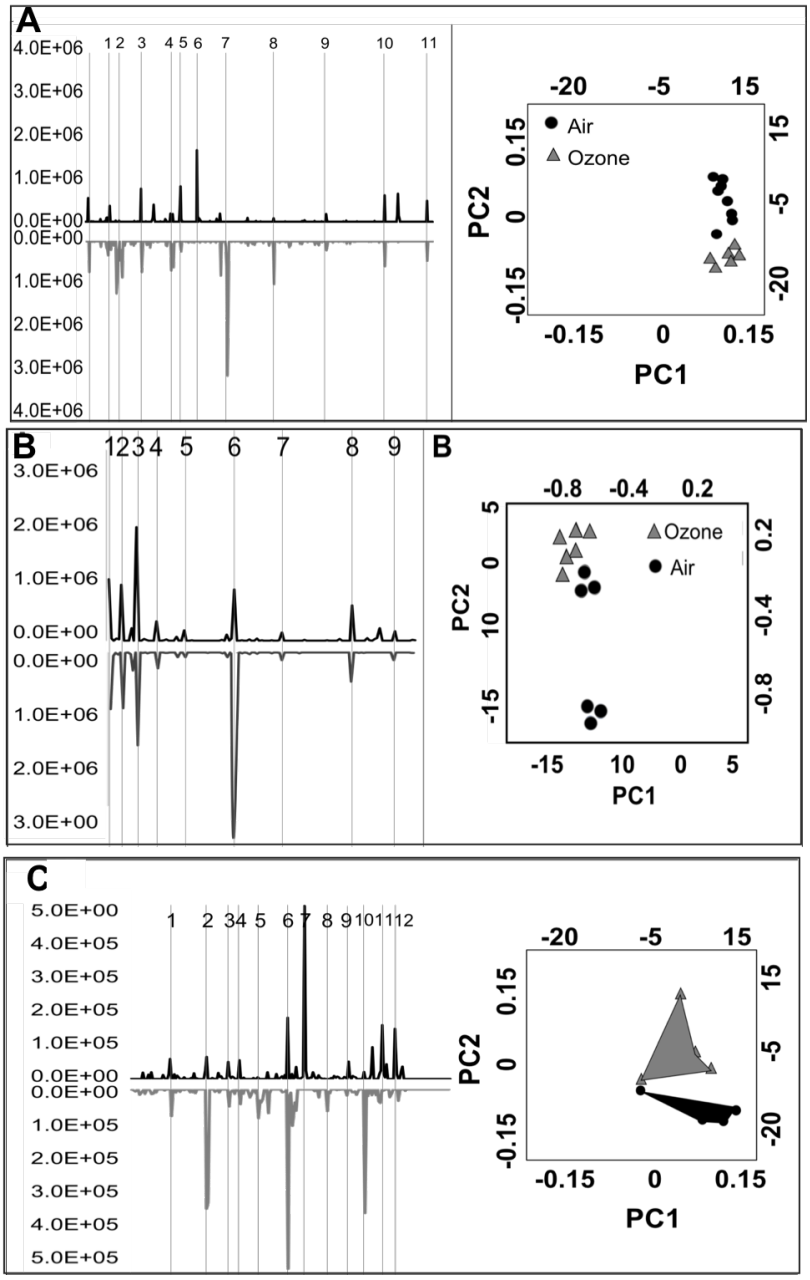


Fig. 7. Ozone impacts odor production of *C. pepo* flowers, whole plants, and pollen. Sample traces plumes from air and ozone-exposed plants are shown, with air-exposed plumes in black and ozone shown as grey. PCA for all samples are shown. **A.**Volatiles emission increased in response to ozone treatment for whole *C. pepo* plants. (log-transformed t-test: t = 2.228, p-value =0.044).**B**. Floral plumes from *C. pepo* exposed to air or ozone showed high variation and no significant difference in concentration (log-transformed t-test: t = 1.182, p-value = 0.059), but PCA showed visually distinct groupings **C.** Ozone and air-exposed pollen plumes were separated in PCA, but there was not a significant increase in abundance of volatiles in ozone-exposed pollen (log-transformed t-test: t=0.441, p-value = 0.8158)

Table 3.
Cucurbita Pepo Volatile ID

	C. Pepo Whole plant
1	1-Hexanol
2	Bromohexane std.
3	2-Heptanone
4	Benzaldehyde
5	6-methyl-5-hepten-2-one
6	2-ethyl-1-hexanol
7	Nonanal
8	1,4-dimethoxybenzene
9	Decanal
10	1,2,4-trimethoxybenzene
11	Indole

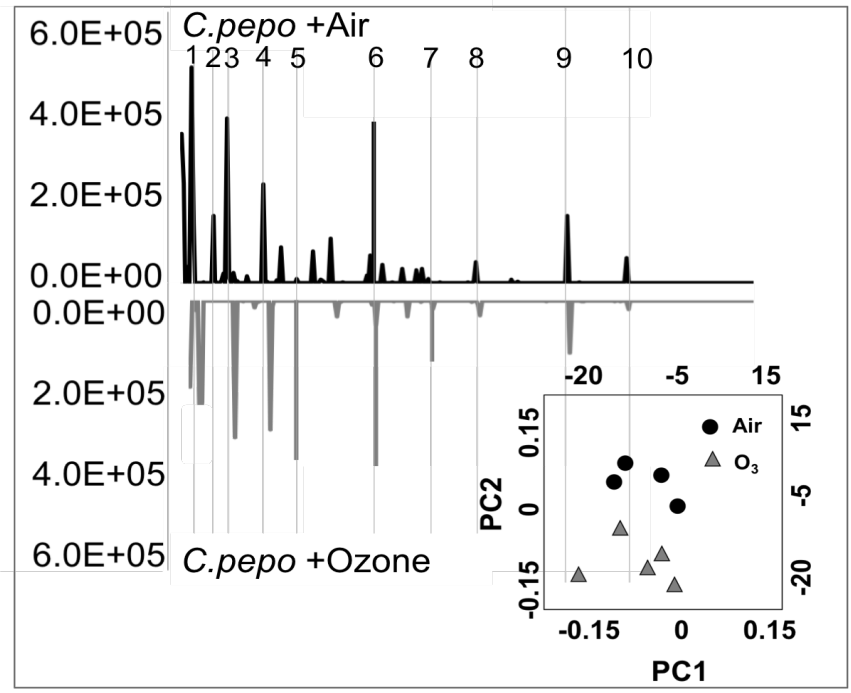
	C. Pepo Flowers ID
1	Bromohexane std.
2	Benzaldehyde
3	1-Octen-3-ol
4	Benzyl alcohol
5	E-E-3,5,-Octadien-2-one
6	1,4,-dimethoxy-benzene
7	Decanal
8	1,2,-Benzisothiazole
9	Isothiocyanate- cyclohexane

	C. Pepo pollen ID
1	Benzaldehyde
2	1-Octen-3-ol
3	Benzyl alcohol
4	E-E-3,5,-Octadien-2-one
5	1,4,-dimethoxy-benzene
6	ethyl-ester octanoic acid
7	Decanal
8	1,2,-Benzisothiazole
9	Isothiocyanate- cyclohexane
10	1,2,4-Trimethoxybenzene
11	6,10-dimethyl-5,9,undecadien-2-one
12	Germacrene D

Table 3. Identification of floral volatiles from ozone-exposed and air-exposed *C.pepo* whole plants, flowers, and pollen. Numbered peaks are identified by the NIST library (R-match > 90%)

Impacts of ozone mixed with C. pepo floral plumes post-emission

In addition to impacting floral plumes by altering production of those plumes, ozone also reacted with *C. pepo* plumes post-emission.



	C. Pepo ozone and air
1	E,E,-6,10-dimethyl-5,9-dodecadien-2-one
2	Heptanal
3	Bromohexane std.
3	Benzaldehyde
4	Benzyl alcohol
5	6-methyl-5-hepten-2-one
6	1,4,-dimethoxy-benzene
7	Decanal
8	1,2,-Benzisothiazole
9	1,2,4-trimethoxybenzene
10	indole

Fig. 8. Ozone altered *C. pepo* plumes: sample trace of *C. pepo* mixed with scrubbed clean air (black) and *C. pepo* plume mixed with 120 ppbv ozone (grey) B. Numbered peaks are identified by the NIST library (R-match > 90%). C. Principal component analysis of original and ozonated headspaces of five *C. pepo* flowers.

Pollinator choice

Squash bees and honey bees responded differently to ozone-exposed vs. air-exposed *C. pepo* plants. While squash bees preferred the air-exposed plants to the ozone-exposed plants, honeybees had no preference.

The *C. pepo* plants used in these behavioral tests exhibit strong evidence of oxidative damage, including yellowing and speckling, as reflected by a decrease in measurements of leaf chlorophyll in leaves of ozone-exposed *C. pepo* plants. Moreover, ozone-exposed plants had a

decrease in number of leaves as well as buds after their exposure to ozone, although plants treated in just clean-air chambers also had a decrease in number of leaves.

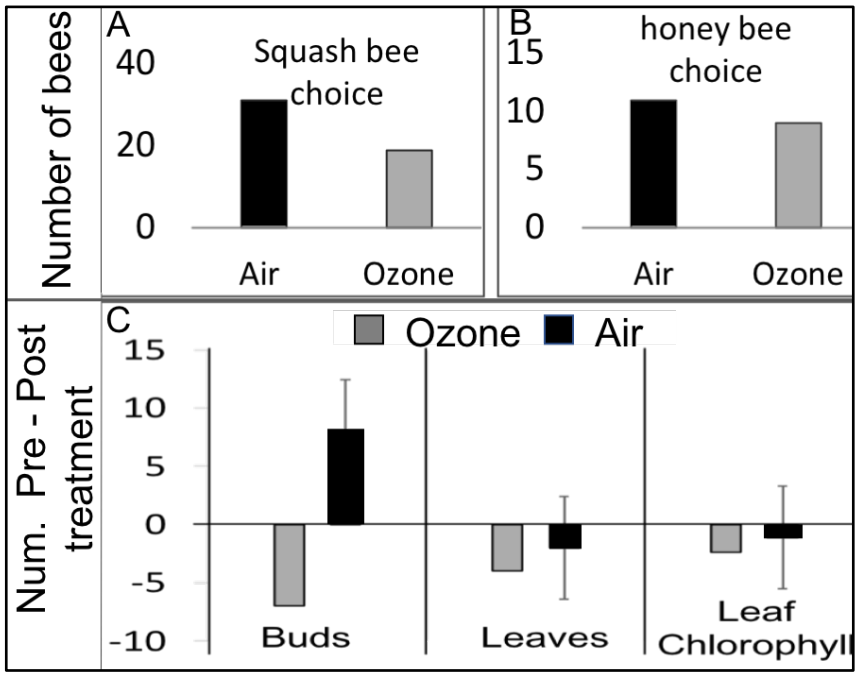


Fig. 9 A. Squash bees preferentially investigated air-exposed *C. pepo* plants more often than ozone-exposed *C. pepo* plants (negative binomial regression: *-0.4969Ozone/Air Choice -0.0069Plant_Pair +0.6659Plant_Pair*Ozone/Air Choice +0.7847, p-value for Ozone/Air TRT* = 0.0192). **B**. Honeybees did not prefer *C. pepo* plants exposed to ozone or to air (negative binomial regression: *-0.4559Ozone/Air Choice +0.021Plant_Pair -0.0641Plant_Pair*Ozone/Air Choice +0.7847, p-value for Ozone/Air TRT: p*-value = 0.718.) **C.** *C. pepo* plants exposed to ozone and used in bee behavior choice tests had greater decrease in number of buds (Wilcoxon: W= 363.4, p-value = 0.00146), number of leaves (W= 221.8, *p*-value = 0.0481) and amount of leaf chlorophyll (W= 265.5 p-value =0.0332).

Squash bee response to ozone-altered C. pepo *plumes*

Peponapis pruinosa were tested for their initial choice for paper flowers emitting either air vs. a floral scent of four *C. pepo* flowers, or air vs. a floral scent of four *C. pepo* flowers mixed with

ozone. Squash bees did not significantly prefer ozone-altered plumes to air, but did prefer plumes mixed only with air to air.

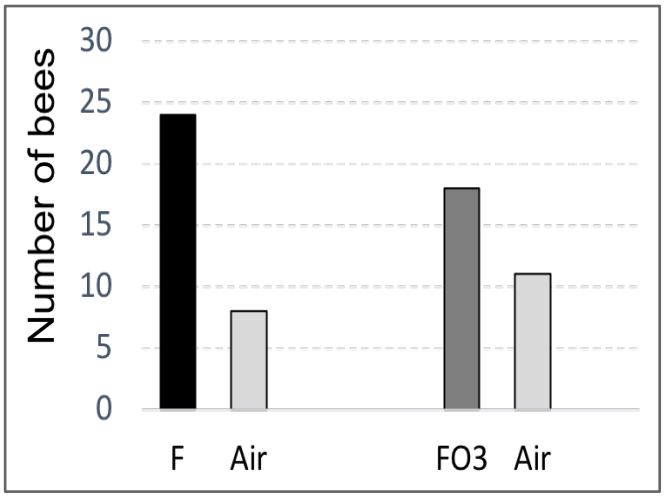


Fig. 10. *Peponapis pruinosa* initially investigate artificial flowers presenting floral scent (F) more often than those emitting air (χ^2 =6.533, *p*-value=.0105, n=51), but do not initially investigate artificial flowers presenting ozone-altered scent (FO3) more often than those emitting air (χ^2 =1.286, *p*-value =0.258, n=34).

DISCUSSION

Ozone exposure was sufficient to cause oxidative damage in all three species

Before assessing ozone-induced alterations in floral signals and rewards, I first ascertained that plants exposed to elevated ozone showed previously identified symptoms of oxidative stress. The highest ozone exposure treatments used for all three plant species had a significant impact on growth and health of plants. Whole 'White Cascade' petunia plants demonstrated a decrease in number of leaves and buds with increasing ozone concentrations and a decrease in flower size (Fig.2). *Nicotiana alata* also had a decrease in number of buds and number of flowers when exposed to ozone and produced smaller flowers (Fig.4) with visible oxidative damage on the sepals (Image 5). Whole *C. pepo* plants exposed to ozone exhibited strong visual evidence of oxidative damage on their leaves (Image 3). Ozonation of *C. pepo* for plants used in bee behavior

assays showed fewer buds relative to air-exposed plants (Fig.9) and strong damage to leaf tissue, as assessed by a decrease in leaf chlorophyll content (Fig.9). In addition to confirming previous studies, which find that ozone-exposure causes a decrease in number of leaves (Morgan et al. 2005) and flowers (Gimeno et al. 2004; Chappelka 2002; Black et al. 2000; Franzaring et al. 2000), these ozone-induced damages to leaf, bud, and flower abundance and presentation may constitute diminished or deteriorated visual signals and impact pollinator preference. Pollinators have been shown to discriminate against flowers based on size, preferring larger to smaller flowers (Martin 2004; Cohen and Shmida et al. 1993). The number of flowers per stem may also influence pollinator choice, with some pollinators preferring stems with more abundant flowers or inflorescences with more umbels (Thompson 2001; Thompson 1988). In addition to affecting flowers' visual signals, oxidative stress also diminished *N. alata*'s tube length, which could decrease the foraging efficiency of pollinators that have evolved a proboscis length best suited to the *N. alata* tube length. In a study on the energy efficiency of foraging *Manduca sexta*, the hawkmoth only experienced a net gain in energy when feeding from *N. alata,* its preferred host flower, losing energy when hovering to feed from flowers with either longer or shorter tube lengths (Haverkamp et al. 2016). Moreover, while the symptoms of oxidative stress manifested in overlapping but different ways in each of the three species, there was sufficient evidence to establish that plants in this study experienced oxidative damage and could thus be further investigated for impacts of oxidative damage on florals signals and rewards. Impacts of ozone on floral signals and rewards, particularly in the context of how these rewards entice pollinator visitation, has only recently begun to be studied (Stabler 2016; Witton 2016).

Ozone impacts production of floral and pollen volatiles

Production of both floral plume abundance and composition were altered when either whole *C. pepo* plants or isolated *N. alata* stems were exposed to ozone--petunia plants were not tested for ozone-stressed volatile release. In accordance with previous work on ozone-stressed whole plants (Vuorinen et al. 2004; Heiden et al. 1999), I found that ozone-exposed *C. pepo* plants had an increase in total volume of floral volatile production relative to air-exposed plants (Fig.7B). In addition, exposing *N. alata* stems to ozone caused a significant increase in the total volume of floral volatiles produced (Fig.5). Ozone exposure did not cause a significant increase in floral volatiles from *C. pepo* flowers as it did for whole *C. pepo* plants, possibly due to the high variation in floral plumes collected from *C. pepo*. *C. pepo* flowers release volatiles in a sudden burst (Ferrari et al. 2006), and the 20-minute floral volatile collections used in this study may have occurred slightly before or after the peak of this burst, leading to strong variations in abundance per flower.

In addition to causing increases in VOC abundance, ozone also had an impact on the composition of floral plumes. This was most noticeable in the case of *C. pep*o flowers exposed to ozone, which showed a strong difference in composition, primarily driven by a more than a 10-fold increase in 1,4-dimethoxybenzene for ozone-exposed *C. pepo* flowers (Fig.6). In *N. alata*, increases in ozone-exposed volatile abundance were not driven by any dramatic increase in one compound. A more detailed analysis of the change in relative ratios of volatiles from ozone-exposed and air-exposed plumes is temporarily delayed: because VOCs were collected via PDMS tubes which both absorb and release compounds, it is not possible to directly quantify differences in the abundance of compounds accurately, and the differences I observed in abundance of compounds and relative ratios between ozone-exposed and air-exposed plumes may be even more dramatic than if compounds had continued to accrue as with tenax collection.

A further analysis of VOCs from *C. pepo* plants is planned for August 2019, collecting volatiles with tenax and assessing changes in ratios of compounds based on those quantifiable samples.

In addition to alterations in relative ratios of compounds in the plume, some compounds were identified only in the plume of ozone-exposed plants (compounds were present in at least 50% of traces from ozone-exposed plants and in no traces from air-exposed plants). These compounds may appear in the plume either because they are formed as a result of plants' oxidative stress or may reflect an increase in the abundance of these compounds above detection thresholds. Both beta-caryophyllene and methyl salicylate were detected only in floral plumes from ozone-exposed *N. alata*. A previous study assessing ozone's impact on VOC production in tobacco found an ~6-fold increase in beta-caryophyllene and a nearly 100-fold increase in methyl salicylate emissions from leaf and stem tissue of ozone-exposed *N. tabacum* (Heiden et al. 1999). While I collected volatiles only from flowers of *N. alata*, the flowers were still attached to the plant and contained sepal and stem portions of the plant. In *C. pepo,* 1-hexanol was found only in *C. pepo* plants not exposed to ozone, but no additional compounds were detected in the plumes of ozone-exposed *C. pepo* flowers relative to air-exposed *C. pepo* flowers.

Ozone did not cause significant differences in the total volume of *C. pepo* pollen volatiles but did lead to differences in pollen odor composition (Fig.7C). Pollen from all ozone-exposed *C. pepo* plants had one compound not present in any of the air-exposed *C. pepo* pollen: 1-octen-3-ol. While 1-octen-3-ol has been identified in scutellaria flowers (Takeoka et al. 2008), oilseed rape flowers (Pham-Delegue et al. 1993), wheat panicles (Birkett et al. 2004), and honey produced by *Apis mellifera* foraging on eucalyptus (Verzera et al. 2001), it is most famously known as a 'mushroom alcohol' (Hung et al. 2014). Because 1-octen-3-ol is a major volatile metabolite produced by mold fungi (Kishimoto et al. 2007), the presence of this compound could

indicate that pollen collected from ozone-exposed host plants was molding, even though this was not visible on the pollen.

Ozone impacts floral signals post-emission

While ozone altered the production of floral odors, it had even more dramatic impacts on plume composition post-emission. In line with previous work, we find that exposing plume post-emission to 120 ppbv ozone reduced the abundance of the majority of floral volatiles in the plume (Li et al. 2016; Farre-Armengol et al. 2016; Cook et al in prep). In addition to decreasing the abundance of the majority of compounds, ozone exposure also altered the ratios of compounds in the plume, which are potentially important for insect host-recognition (Bruce 2005; Bruce and Picketts 2011). The change in ratios is caused when ozone has a faster reaction rate with some compounds relative to others. Benzaldehyde for *Petunia* 'White Cascade' and *C. pepo*, 1,2,4-trimethoxybenzene for *C. pepo* plants and pollen, and eucalyptol for *N. alata* are largely unreactive with ozone and so do not become reduced after mixing with ozone, even as other compounds in the plume decrease in abundance.

Moreover, ozone also altered plumes by adding new compounds: at least two previously-identified secondary volatiles, long-lived reaction products of tropospheric oxidants and primary floral volatiles, were detected in the ozone-exposed plumes of the three flower species. Ozone-mixed plumes of *C. pepo, N. alata,* and *Petunia* 'White Cascade' contained 6-Methyl-5-hepten-2-one, an atmospheric reaction product of linalool and tropospheric oxidants (Shu et al. 1997), and ozonated plumes of petunia further contained E-6-10-dimethyl-5,9-undecadien-2-one, or geranyl acetone, another previously identified secondary volatile (Fruekilde et al. 1998).

Ozone altered floral nectar and pollen rewards, but responses were mixed and constrained

Ozone exposure led to changes in the production of floral rewards in all three species, but these alterations varied between species and were relatively constrained. Ozone increased the production of nectar in 'White Cascade' when whole plants were exposed and in 'multiflora' when only flowers were exposed to ozone (Fig.1A). Ozone-exposure also increased production of nectar volume in *C. pepo* (Fig6A), but it did not have an effect on nectar volume of *N. alata* (Fig4A). The species-specific response in plant nectar production to elevated ozone corroborates and clarifies the same trend documented in previous field studies (Witton 2016). This increase in nectar volume for ozone-exposed plants may be offset by decreases in nectar sugar concentration, with whole-plant ozone-exposed *Petunia* 'White Cascade' flowers showing a decrease in nectar sugar concentration with increasing ozone concentration (Fig.1D). While neither *N. alata* nor *C. pepo* had a significant decrease in nectar sugar concentration with increasing ozone concentration, ozone-exposed *N. alata* had a significant decrease in the amount of fructose in its nectar, and ozone had a similar but non-significant effect on *C. pepo* nectar composition (Fig.4H; Fig.6B). Changes to nectar sugar composition could impact pollinator perception of the rewarding flower, as nectar sugar composition is typically very stable (Galetto and Bernardello 2002) and is a strong predictor of pollinator type (Baker and Baker 1990; Dupont et al. 2004).

In contrast to our prediction, ozone did not cause a significant decrease in pollen mass in any of the three flower species: indeed, ozone-exposed 'multiflora' petunia flowers showed a significant increase in pollen production with increasing ozonation (Fig.1B). This may indicate a strong investment in reproduction for oxidant-stressed plants, although this would be a poor investment if pollen is made inviable by ozone. Further analysis of *C. pepo* pollen found that flowers exposed to ozone at least five days prior to pollen collection had an increased C:N ratio (Fig.6E), likely reflecting a decline in total protein content for pollen. *C. pepo's* delayed response

to effects on pollen indicates that ozone-exposure may need to occur early during pollen formation in order to affect a plant's allocation of nutrients to pollen.

However, while the abundance of nectar, nectar sugar composition, and the C:N ratio of pollen were altered by exposure to ozone for some species, this response was inconsistent and relatively constrained compared to very visible damage to plant leaf tissue (Image 4) and dramatic decreases observed both in the number of leaves (Fig.2C; Fig.9C), number of flowers (Fig.4A), and number of buds (Fig.2C; Fig.4B; Fig.9C) in some ozone-exposed plants relative to air-exposed plants. The overall relatively small changes to plant pollen and nectar in the face of dramatic impacts to plant structures indicates that plants tightly constrain their production of nectar and pollen. Given that nectar and pollen quality are important to incentivize conspecific pollination, and that pollen transfer is the basis of reproduction in these plant species, this conservation of nectar and pollen even in the face of decreasing numbers of flowers could highlight a strategy meant to maximize reproduction, investing in plant-pollinator mutualism even as the plant undergoes strong oxidative stress.

Pollinator choice in response to pre and post-emission ozone-exposed plumes

The production of floral rewards and floral signals is altered by exposure to ozone, but do such changes affect pollinator choice? We find that ozone-exposed squash plants are significantly less attractive to female squash bees when compared to squash plants exposed only to clean air (Fig.9A). Both floral volatiles and pollen odors were altered in ozone-exposed *C. pepo* flowers (Fig.7), which may drive pollinator choice in this case. Since elevated ozone resulted in a higher volume of floral scents emitted from whole *C. pepo* plants, it might be assumed that ozonation would make plants more attractive to pollinators, as an increase in the production of odors could 'amplify' the signal that the plants produce. This prediction would seem particularly likely in the

case of ozonated *C .pepo* flowers which had a dramatic increase in 1,4-dimethoxybenzene, an attractive compound to the pollinator *P. pruinosa* (Adler and Theis 2012). However, increasing concentration does not necessarily increase the attractiveness of a signal to a pollinator: when 1,4-dimethoxybenzene was artificially enhanced at the flower, it did not make the plume more attractive to pollinators (Adler and Thies 2012). More generally, plumes of ozone-elevated VOCs have not been found to increase attraction to mutualist parasitoid insects, even when VOCs elevated by herbivore attacks do (Hlaking et al. 2016; Vuorinen et al. 2004). In addition to ozone-induced changes in olfactory cues, ozone damage to visual cues may also have played a role in *P. pruinosa's* preference for non-ozonated *C. pepo* plants. Ozone-exposed *C. pepo* plants were visibly damaged, with squash leaves exhibiting flecking and yellowing, as quantified via decrease in leaf chlorophyll, and ozonated plants also had fewer buds and leaves relative to air-exposed plants (Fig.9C).

In contrast to *P. pruinosa's* discrimination between ozone-exposed and air exposed squash plants, preliminary results find no difference in preference for *A. mellifera,* although overall *A. mellifera* did not find either option very enticing, leading to an overall low response rate. If *A. mellifera's* apparent lack of discrimination is not just an artefact of low sample size, it indicates that specialists may be more discriminatory than generalists against host-plants that are ozone-damaged or otherwise anthropogenically perturbed.

Because *P. pruinosa* still chose to initially investigate ozone-exposed *C. pepo* plants approximately 30% of the time relative to air-exposed plants, *P. pruinosa's* discrimination against ozonated plants does not necessarily mean that insects would be not visit ozone-damaged *C. pepo* plants if there were not undamaged alternatives. If this is the case, avoiding ozone-damaged *C. pepo* plants may benefit *P. pruinosa,* if oxidative stress is indicative of poor rewards. Moreover, *P. pruinosa's* discrimination against ozone-exposed individuals may benefit

plants at a population level: if ozone causes pollen to be less viable, then the conspecifics of an ozone-damaged individual benefit if that damaged individual is not visited by pollinators and given the opportunity to spread inviable pollen.

While *P. pruinosa* choosing between ozone-exposed and air exposed *C. pepo* plants responded to ozonated plants ~30% of the time, they did not retain this cantlet of attraction to *C. pepo* plumes mixed with ozone post-emission: while *P. pruinosa* preferred artificial flowers emitting the *C. pepo* plume to clean air, they did not discriminate between the ozone-exposed *C. pepo* plume and air (Fig.10). In line with their stringent discrimination for host flowers, *P. pruinosa* had only low responsiveness to artificial flowers without *C. pepo* plumes, so bees were not assessed for their response to ozone vs. air. The loss of attraction to the ozone-exposed plume may therefore be due either to an avoidance of ozone itself or to the insect's loss of recognition due to ozone-induced changes in the squash plume. However, previous studies on a range of taxonomically diverse insects, including a specialist (the beetle *Acalymma vittatum*), find that ozone was not a deterrent to insects, while exposed plumes were less attractive to these foraging insects (Fuentes et al. 2013; Farre-armengol et al. 2016; Li et l 2016; Cook et al. 2019 in prep)

Learning ozone-altered plumes pre and post emission

Ozone exposure to plants and ozone reaction with emitted floral plumes causes both to be less attractive to the pollinator *P. pruinosa*. Thus, anthropogenically elevated ozone could degrade plant-pollinator communication and thereby endanger pollinators that rely on flowers as food sources and plants that depend on pollinators to sexually reproduce. However, insect learning may help mitigate the effects of ozone-induced variability in floral signals. Learning plumes that are altered by ozoneas they move through the troposphere poses several challenges to foraging insects: insects would have to learn these plumes decoupled from plant rewards, as it is only as

the plume moves downwind of the flower that it becomes altered by ozone. Furthermore, plumes will become more altered by ozone as they move away from the flower, setting up a gradient of increased alterations as the plume leaves the emission point and mixes in the troposphere.However, despite the difficulties to learning presentedby this system, a previous study finds that the hawkmoth *M. sexta* can readily learn floral plumes mixed with ozone post-emission, associating the altered plumes with sucrose rewards (Cook et al. in prep). This was achieved even in a scenario where rewards and ozonated plumes are decoupled, as they would be in a polluted environment where plumes mix with ozone as they move downwind of the rewarding host plant. *Manduca sexta's* ability to learn the ozone-altered plume spatially and temporally decoupled from floral rewards is encouraging and indicates that insects may have the potentialto plastically respond to altered plumes under high-ozone pollution events, although further research on this topic is required.

In contrast to the difficulties presented by ozone-alterations of plumes moving downwind of flowers, learning floral plumes emitted from oxidant-stressed plants would seem less challenging. Pollinators could simply associate floral signals altered by ozone exposure with floral rewards (potentially also altered by ozone exposure) at the flower. However, there is a complication to this ostensibly simple learning: pollinators would have to find ozone-damaged plants sufficiently attractive to forage on them initially. Unlike the case in which floral plumes are intact at the flower and become increasingly altered as they move downwind, the floral plumes which have been altered due to plant ozone-exposure are less attractive at the release point. However, if pollinators find ozone-damaged plants less attractive but still sufficiently attractive enough to investigate, as in the case of *P. pruinosa* visiting ozone-damaged *C. pepo* plants ~30% of the time, then the pollinator would have the opportunity to learn the changed floral cue. Whether the pollinator learns to avoid or further investigate ozone-damaged flowers

will then depend on the quality of rewards, giving pollinators a powerful tool to cope with both plume variation and reward variation induced by plant ozone-exposure.

While the ability of specialist pollinators such as *P. pruinosa* to learn olfactory cues is not as well demonstrated as it is in the case of generalist pollinators, some of which have prodigious learning skills (Hammer and Menzel 1995; Sommerlandt et al. 2014; Riveros & Gronenberg 2009), there is emerging evidence that specialists may learn to rely more heavily on some visual cues (Milet-Pinheiro et al. 2012) or may learn expanded olfactory (Burger et al. 2010) cues. Thus, even specialist pollinators may use learning to cope with ozone-induced alterations to floral signals and rewards.

Broader impacts and future directions

Floral signals and rewards have been integral to maintaining plant-pollinator relationships over evolutionary timescales. Anthropogenically elevated tropospheric air pollution could interfere with plant-pollinator interactions by rapidly altering both floral signals and rewards. Under conditions of increased air pollution, relationships between plants and pollinators may be strengthened, as in the case of increasing nectar volume production from ozone-exposed plants, or weakened if produced floral plumes or floral plumes in transmission are not recognized by foraging pollinators. Moreover, the strengthening and weakening of relationships may be strongly dependent on the plant's response to elevated ozone and the resiliency of its floral plume to ozone-degradation. Thus, while changes to relationships between plants and pollinators are likely to be highly specific to the plant and pollinator involved, ozone is likely to affect pollination visitation in a community. This study does not explicitly test whether pollinators' ability to learn ozone-exposed flowers is disrupted by the changes to floral rewards, and outcomes of such tests could better elucidate how ozone may disrupt or change specific plant-

pollinator interactions. However, this work does establish that ozone can alter floral signals and rewards and that these alterations affect pollinator visitation, opening the door for further investigative work on the effects of ozone on plant-pollinator communication.

Citations

Ashmore, M. R. (2005). Assessing the future global impacts of ozone on vegetation. *Plant, Cell & Environment*, *28*(8), 949-964.

Baker, H. G., & Baker, I. (1990). The predictive value of nectar chemistry to the recognition of pollinator types. *Israel Journal of Plant Sciences*, *39*(1-2), 157-166.

Bernardello G., Galetto L., Anderson G. J. (2000) Floral nectary structure and nectar chemical composition of some species from Robinson Crusoe Island (Chile). Canad. J. Bot. 78: 862–872.

Birkett, M. A., Bruce, T. J., Martin, J. L., Smart, L. E., Oakley, J. O. N., & Wadhams, L. J. (2004). Responses of female orange wheat blossom midge, Sitodiplosis mosellana, to wheat panicle volatiles. *Journal of chemical ecology*, *30*(7), 1319-1328.

Bitterman, M. E. 1976: Incentive contrast in honey bees. Science 192, 380—382.

Blande JD, Tiiva P, Oksanen E, Holopainen JK. Emission of herbivore-induced volatile terpenoids from two hybrid aspen (*Populus tremula* × *tremuloides*) clones under ambient and elevated ozone concentrations in the field. Global Change Biology. 2007b;13:2538–2550.

Brodschneider, R., & Crailsheim, K. (2010). Nutrition and health in honey bees. *Apidologie*, *41*(3), 278-294.

Bruce, T. J., Wadhams, L. J., & Woodcock, C. M. (2005). Insect host location: a volatile situation. *Trends in plant science*, *10*(6), 269-274.

Bruce, T. J., & Pickett, J. A. (2011). Perception of plant volatile blends by herbivorous insects–finding the right mix. *Phytochemistry*, *72*(13), 1605-1611.

Burger, H., Dötterl, S., & Ayasse, M. (2010). Host-plant finding and recognition by visual and olfactory floral cues in an oligolectic bee. *Functional Ecology*, *24*(6), 1234-1240.

Buchanan, G. M. & Bitterman, M. E. 1988: Learning in honeybees as a function of amount and frequency of reward. Anim. Learn. Behav. 16, 247—255.

Cardoza YJ, Harris GK, Grozinger CM (2012) Effects of soil quality enhancement on pollinator-plant interactions. Psyche 2012:581458.

Cnaani, J., Thomson, J. D., & Papaj, D. R. (2006). Flower choice and learning in foraging bumblebees: effects of variation in nectar volume and concentration. Ethology, 112(3), 278-285.

Cohen, D., & Shmida, A. (1993). The evolution of flower display and reward. In *Evolutionary biology* (pp. 197-243). Springer, Boston, MA.

Cook, S. M., Bartlet, E., Murray, D. A., & Williams, I. H. (2002). The role of pollen odour in the attraction of pollen beetles to oilseed rape flowers. *Entomologia experimentalis et applicata*, *104*(1), 43-50.

Couvillon, P. A. & Bitterman, M. E. 1993: Learning in honeybees as a function of amount of reward: further experiments with color. Anim. Learn. Behav. 21, 23—28.
Cui, H., Su, J., Wei, J., Hu, Y., & Ge, F. (2014). Elevated O 3 enhances the attraction of whitefly-infested tomato plants to Encarsia formosa. *Scientific reports*, *4*, 5350.
Dobson, H. E. M. (1987). Role of flower and pollen aromas in host-plant recognition by solitary bees. *Oecologia*, *72*(4), 618-623.
Dobson, H. E. D., Groth, I., & Bergström, G. (1996). Pollen advertisement: Chemical contrasts between whole-flower and pollen odors. *American Journal of Botany*, *83*(7), 877-885.
Dötterl, S., & Schäffler, I. (2007). Flower scent of floral oil-producing Lysimachia punctata as attractant for the oil-bee Macropis fulvipes. *Journal of chemical ecology*, *33*(2), 441-445.
Dupont, Y. L., Hansen, D. M., Rasmussen, J. T., & Olesen, J. M. (2004). Evolutionary changes in nectar sugar composition associated with switches between bird and insect pollination: The Canarian bird-flower element revisited. *Functional Ecology*, *18*(5), 670-676.
Eberhard, S. H., Hrassnigg, N., Crailsheim, K., & Krenn, H. W. (2007). Evidence of protease in the saliva of the butterfly Heliconius melpomene (L.)(Nymphalidae, Lepidoptera). *Journal of Insect Physiology*, *53*(2), 126-131.
Elkiey, T., & Ormrod, D. P. (1979a). Leaf diffusion resistance responses of three petunia cultivars to ozone and/or sulfur dioxide. *Journal of the Air Pollution Control Association*, *29*(6), 622-625.
Elkiey, T., & Ormrod, D. P. (1979ba). Ozone and/or sulphur dioxide effects on tissue permeability of petunia leaves. *Atmospheric Environment (1967)*, *13*(8), 1165-1168.
Erhardt, A., & Baker, I. (1990). Pollen amino acids—an additional diet for a nectar feeding butterfly?. *Plant Systematics and Evolution*, *169*(1-2), 111-121.
Faoro, F., & Iriti, M. (2009). Plant cell death and cellular alterations induced by ozone: key studies in Mediterranean conditions. *Environmental Pollution*, *157*(5), 1470-1477.
Feng Z, Kobayashi K, Ainsworth EA (2008) Impact of elevated ozone concentration on growth, physiology, and yield of wheat (Triticum aestivum L.): a meta-analysis. Global Change Biology, 14, 2696–2708.
Fiscus E. L., Booker F. L., Burkey K. O. (2005) Crop responses to ozone: uptake, modes of action, carbon assimilation and partitioning. Plant, Cell and Environment, 28, 997–1011.

Fruekilde, P., Hjorth, J., Jensen, N. R., Kotzias, D., & Larsen, B. (1998). Ozonolysis at vegetation surfaces: a source of acetone, 4-oxopentanal, 6-methyl-5-hepten-2-one, and geranyl acetone in the troposphere. *Atmospheric Environment*, *32*(11), 1893-1902.
Fuhrer, J. (2009). Ozone risk for crops and pastures in present and future climates. *Naturwissenschaften*, *96*(2), 173-194.
Gilbert, L. E. (1972). Pollen feeding and reproductive biology of Heliconius butterflies. *Proceedings of the National Academy of Sciences*, *69*(6), 1403-1407.
Giurfa, M. A. R. T. I. N., & Lehrer, M. I. R. I. A. M. (2001). Honeybee vision and floral displays: from detection to close-up recognition. *Cognitive ecology of pollination*, 61-82.
Gould, J. L. (1985). How bees remember flower shapes. *Science*, *227*(4693), 1492-1494.
Gumbert, A. (2000). Color choices by bumble bees (Bombus terrestris): innate preferences and generalization after learning. *Behavioral Ecology and Sociobiology*, *48*(1), 36-43.
Hanley, M. E., Franco, M., Pichon, S., Darvill, B., & Goulson, D. (2008). Breeding system, pollinator choice and variation in pollen quality in British herbaceous plants. *Functional Ecology*, *22*(4), 592-598.
Hanson, G. P., Thorne, L., & Addis, D. H. (1975). Ozone sensitivity of Petunia hybrida vilm. as related to physiological age. *J. Am. Soc. Hortic. Sci.;(United States)*, *100*(2).

Hauglustaine, D. A., & Brasseur, G. P. (2001). Evolution of tropospheric ozone under anthropogenic activities and associated radiative forcing of climate. *Journal of Geophysical Research: Atmospheres*, *106*(D23), 32337-32360.
Heath, R. L. (1980). Initial events in injury to plants by air pollutants. *Annual review of plant physiology*, *31*(1), 395-431.
Heath, R. L. (1994). Possible mechanisms for the inhibition of photosynthesis by ozone. *Photosynthesis Research*, *39*(3), 439-451.
Herrera, C. M., Pérez, R., & Alonso, C. (2006). Extreme intraplant variation in nectar sugar composition in an insect-pollinated perennial herb. *American Journal of Botany*, *93*(4), 575-581.
Heiden A.C, Hoffmann, T., Kahl, J., Kley, D., Klockow, D., Langebartels, C. & Wildt, J. (1999). Emission of volatile organic compounds from ozone-exposed plants. *Ecological Applications*, *9*(4), 1160-1167.
Hill, P. S., Hollis, J., & Wells, H. (2001). Foraging decisions in nectarivores: unexpected interactions between flower constancy and energetic rewards. *Animal Behaviour*, *62*(4), 729-737.
Holopainen, J. K., & Gershenzon, J. (2010). Multiple stress factors and the emission of plant VOCs. *Trends in plant science*, *15*(3), 176-184.
Hung, R., Lee, S., & Bennett, J. W. (2014). The effects of low concentrations of the enantiomers of mushroom alcohol (1-octen-3-ol) on Arabidopsis thaliana. *Mycology*, *5*(2), 73-80.
Iriti, M., & Faoro, F. (2009). Ozone-induced changes in plant secondary metabolism. In *Climate Change and Crops* (pp. 245-268). Springer, Berlin, Heidelberg.
Julier, Esther H., & Roulston, T. A. H. (2009). Wild bee abundance and pollination service in cultivated pumpkins: farm management, nesting behavior and landscape effects. *Journal of Economic Entomology*, *102*(2), 563-573.
Khaling, E., Papazian, S., Poelman, E. H., Holopainen, J. K., Albrectsen, B. R., & Blande, J. D. (2015). Ozone affects growth and development of Pieris brassicae on the wild host plant Brassica nigra. *Environmental pollution*, *199*, 119-129.
Keller, I., Fluri, P., & Imdorf, A. (2005). Pollen nutrition and colony development in honey bees: part 1. *Bee world*, *86*(1), 3-10.
Kitaoka, T. K., & Nieh, J. C. (2009). Manuscript in preparation for Behavioral Ecology and Sociobiology Bumble bee pollen foraging regulation: role of pollen quality, storage levels, and odor. *Behavioral Ecology and Sociobiology*, *63*(4), 501-510.
Kitaoka TK, Nieh JC (2008) Bumble bee pollen foraging regulation: Role of pollen quality, storage levels, and odor. Behav Ecol Sociobiol 63(4):501–510. 28.
Kishimoto, K., Matsui, K., Ozawa, R., & Takabayashi, J. (2007). Volatile 1-octen-3-ol induces a defensive response in Arabidopsis thaliana. *Journal of General Plant Pathology*, *73*(1), 35-37.
Klein, A. M., Vaissiere, B. E., Cane, J. H., Steffan-Dewenter, I., Cunningham, S. A., Kremen, C., & Tscharntke, T. (2006). Importance of pollinators in changing landscapes for world crops. *Proceedings of the royal society B: biological sciences*, *274*(1608), 303-313.
Konzmann S, Lunau K (2014) Divergent rules for pollen and nectar foraging bumblebees–a laboratory study with artificial flowers offering diluted nectar substitute and pollen surrogate. PLoS One 9(3):e91900.
Krupa, S. V., & Manning, W. J. (1988). Atmospheric ozone: formation and effects on vegetation. *Environmental Pollution*, *50*(1-2), 101-137.
Krupnick, G. A., Weis, A. E., & Campbell, D. R. (1999). The consequences of floral herbivory for pollinator service to Isomeris arborea. *Ecology*, *80*(1), 125-134.

Kunze, J., & Gumbert, A. (2001). The combined effect of color and odor on flower choice behavior of bumble bees in flower mimicry systems. *Behavioral Ecology*, *12*(4), 447-456.
Lee, Y. & Bitterman, M. E. 1990: Learning in honeybees as a function of amount of reward: control of delay. Anim. Learn. Behav. 18, 377—386.
Linsley E.G. (1958) The ecology of solitary bees, Hilgardia 27, 543–599.
McFrederick, Q. S., Kathilankal, J. C., & Fuentes, J. D. (2008). Air pollution modifies floral scent trails. *Atmospheric Environment*, *42*(10), 2336-2348.
Martin, N. H. (2004). Flower size preferences of the honeybee (Apis mellifera) foraging on Mimulus guttatus (Scrophulariaceae).
Milet-Pinheiro, P., Ayasse, M., Schlindwein, C., Dobson, H. E., & Dötterl, S. (2012). Host location by visual and olfactory floral cues in an oligolectic bee: innate and learned behavior. *Behavioral Ecology*, *23*(3), 531-538.
Muth F., Papaj, D. R., & Leonard, A. S. (2016). Bees remember flowers for more than one reason: pollen mediates associative learning. *Animal behaviour*, *111*, 93-100.
Mery, F., & Kawecki, T. J. (2004). An operating cost of learning in Drosophila melanogaster. *Animal Behaviour*, *68*(3), 589-598.
Morgan, P. B., Ainsworth, E. A., & Long, S. P. (2003). How does elevated ozone impact soybean? A meta-analysis of photosynthesis, growth and yield. *Plant, Cell & Environment*, *26*(8), 1317-1328.
Ollerton, J., Winfree, R., & Tarrant, S. (2011). How many flowering plants are pollinated by animals?. *Oikos*, *120*(3), 321-326.
Nouchi, I., Mayumi, H., & Yamazoe, F. (1984). Foliar injury response of petunia and kidney bean to simultaneous and alternate exposures to ozone and PAN. *Atmospheric Environment (1967)*, *18*(2), 453-460.
Pernal, S. F., & Currie, R. W. (2002). Discrimination and preferences for pollen-based cues by foraging honeybees, Apis mellifera L. *Animal Behaviour*, *63*(2), 369-390.
Pham-Delegue, M. H., Bailez, O., Blight, M. M., Masson, C., Picard-Nizou, A. L., & Wadhams, L. J. (1993). Behavioural discrimination of oilseed rape volatiles by the honeybee Apis mellifera L. *Chemical senses*, *18*(5), 483-494.
Pinto, D. M., Blande, J. D., Nykänen, R., Dong, W. X., Nerg, A. M., & Holopainen, J. K. (2007). Ozone degrades common herbivore-induced plant volatiles: does this affect herbivore prey location by predators and parasitoids?. *Journal of chemical ecology*, *33*(4), 683-694.
Pitcher, L. H., Brennan, E., Hurley, A., Dunsmuir, P., Tepperman, J. M., & Zilinskas, B. A. (1991). Overproduction of petunia chloroplastic copper/zinc superoxide dismutase does not confer ozone tolerance in transgenic tobacco. *Plant physiology*, *97*(1), 452-455.
Potts, S. G., Biesmeijer, J. C., Kremen, C., Neumann, P., Schweiger, O., & Kunin, W. E. (2010). Global pollinator declines: trends, impacts and drivers. *Trends in ecology & evolution*, *25*(6), 345-353.
Raine, N. E., & Chittka, L. (2011). Flower constancy and memory dynamics in bumblebees (Hymenoptera: Apidae: Bombus). *Entomol Gen*.
Rich, S. (1964). Ozone damage to plants. *Annual Review of Phytopathology*, *2*(1), 253-266.
Robertson, A. W., Mountjoy, C., Faulkner, B. E., Roberts, M. V., & Macnair, M. R. (1999). Bumble bee selection of Mimulus guttatus flowers: the effects of pollen quality and reward depletion. *Ecology*, *80*(8), 2594-2606.
Roulston T. H., Cane J. H., Buchmann S. L. 2000. What governs protein content of pollen: pollinator preferences, pollen-pistil interactions, or phylogeny? Ecological Monographs 70:617–643.

Sandermann, H., Ernst, D., Heller, W., & Langebartels, C. (1998). Ozone: an abiotic elicitor of plant defence reactions. *Trends in Plant Science*, *3*(2), 47-50.
Sandermann, H. (2008). Ecotoxicology of ozone: bioactivation of extracellular ascorbate. *Biochemical and biophysical research communications*, *366*(2), 271-274.
Schemske, D. W., & Ågren, J. (1995). Deceit pollination and selection on female flower size in Begonia involucrata: an experimental approach. *Evolution*, *49*(1), 207-214.
Shu, Y., Kwok, E. S., Tuazon, E. C., Atkinson, R., & Arey, J. (1997). Products of the gas-phase reactions of linalool with OH radicals, NO3 radicals, and O3. *Environmental science & technology*, *31*(3), 896-904.
Smith, B. D. (1997). The initial domestication of Cucurbita pepo in the Americas 10,000 years ago. *Science*, *276*(5314), 932-934.
Somme, L., Vanderplanck, M., Michez, D., Lombaerde, I., Moerman, R., Wathelet, B., ... & Jacquemart, A. L. (2015). Pollen and nectar quality drive the major and minor floral choices of bumble bees. *Apidologie*, *46*(1), 92-106.
Takeoka, G. R., Dao, L., Rodriguez, D. M., & Patterson, R. (2008). Headspace volatiles of Scutellaria californica A. Gray flowers. *Journal of Essential Oil Research*, *20*(2), 169-171.
Theis, N., & Adler, L. S. (2012). Advertising to the enemy: enhanced floral fragrance increases beetle attraction and reduces plant reproduction. *Ecology*, *93*(2), 430-435.
Thomson, J. D. (1988). Effects of variation in inflorescence size and floral rewards on the visitation rates of traplining pollinators of Aralia hispida. *Evolutionary Ecology*, *2*(1), 65-76.
Thompson, J. D. (2001). How do visitation patterns vary among pollinators in relation to floral display and floral design in a generalist pollination system?. *Oecologia*, *126*(3), 386-394.
Vaudo, A. D., Patch, H. M., Mortensen, D. A., Tooker, J. F., & Grozinger, C. M. (2016). Macronutrient ratios in pollen shape bumble bee (Bombus impatiens) foraging strategies and floral preferences. *Proceedings of the National Academy of Sciences*, *113*(28), E4035-E4042.
Verzera, A., Campisi, S., Zappalà, M., & Bonaccorsi, I. (2001). SPME-GC-MS analysis of honey volatile components for the characterization of different floral origin. *American laboratory*, *33*(15), 18-23.
Vingarzan, R. (2004). A review of surface ozone background levels and trends. *Atmospheric Environment*, *38*(21), 3431-3442.
Vuorinen, T., Nerg, A. M., & Holopainen, J. K. (2004). Ozone exposure triggers the emission of herbivore-induced plant volatiles, but does not disturb tritrophic signalling. *Environmental Pollution*, *131*(2), 305-311.
Waddington, K. D., & Gottlieb, N. (1990). Actual vs perceived profitability: a study of floral choice of honey bees. *Journal of Insect Behavior*, *3*(4), 429-441.
Waser, N. M. (1986). Flower constancy: definition, cause, and measurement. *The American Naturalist*, *127*(5), 593-603.
Wilson, P., & Stine, M. (1996). Floral constancy in bumble bees: handling efficiency or perceptual conditioning?. *Oecologia*, *106*(4), 493-499.
Winfree, R., Gross, B. J., & Kremen, C. (2011). Valuing pollination services to agriculture. *Ecological Economics*, *71*, 80-88.
Wright, G. A., & Schiestl, F. P. (2009). The evolution of floral scent: the influence of olfactory learning by insect pollinators on the honest signalling of floral rewards. *Functional Ecology*, *23*(5), 841-851.