EFFECTS OF OZONE ON PLANTS AND PLANT-INSECT INTERACTIONS

Dissertation zur Erlangung des naturwissenschaftlichen
Doktorgrades der Julius-Maximilians-Universität Würzburg

vorgelegt von

Laura Maria Ribeiro Duque

geboren in Avelãs de Caminho, Portugal

Würzburg, 2022

Date of submission/Eingereicht am: 11/05/2022

Members of the doctoral comitee/Mitglieder der Promotionskommission:

Chairperson/Vorsitzender: Prof. Dr. Charlotte Förster

Referee/Gutachter: Prof. Dr. Ingolf Steffan-Dewenter

Referee/Gutachter: Dr. Erik H. Poelman

Date of defence/Tag des Promotionskolloquiums: 27/06/2022

Date of receipt of certificates/Doktorurkunde ausgehändigt am:

Equipped with his five senses, man explores the universe around him and calls the adventure Science.

Edwin Hubble

Table of Contents

Chapter I

Chapter II

Chapter III

Chapter IV

Chapter V

Affidavit

I hereby declare that my thesis entitled: „**Effects of ozone on plants and plant-insect interactions**" is the result of my own work. I did not receive any help or support from commercial consultants. All sources and / or materials applied are listed and specified in the thesis.

Furthermore I verify that the thesis has not been submitted as part of another examination process neither in identical nor in similar form.

Besides I declare that if I do not hold the copyright for figures and paragraphs, I obtained it from the rights holder and that paragraphs and figures have been marked according to law or for figures taken from the internet the hyperlink has been added accordingly.

Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, die Dissertation: „**Effects of ozone on plants and plant-insect interactions**", eigenständig, d. h. insbesondere selbständig und ohne Hilfe eines kommerziellen Promotionsberaters, angefertigt und keine anderen, als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben.

Ich erkläre außerdem, dass die Dissertation weder in gleicher noch in ähnlicher Form bereits in einem anderen Prüfungsverfahren vorgelegen hat.

Weiterhin erkläre ich, dass bei allen Abbildungen und Texten bei denen die Verwertungsrechte (Copyright) nicht bei mir liegen, diese von den Rechtsinhabern eingeholt wurden und die Textstellen bzw. Abbildungen entsprechend den rechtlichen Vorgaben gekennzeichnet sind sowie bei Abbildungen, die dem Internet entnommen wurden, der entsprechende Hypertextlink angegeben wurde.

Würzburg, 11/05/2022

Laura Maria Ribeiro Duque

Acknowledgements

As in all things in life, this work would not be possible without the contribution of several players, which I wish to acknowledge and thank.

I would like to thank the old **me**, for deciding that any time in one's life is good for starting a new project and for pushing through the rougher times.

A big thank you to **Prof. Dr. Ingolf Steffan-Dewenter** for welcoming me in his team and for giving me the means to work. Thank you for advising me along this journey, while giving me space to explore my own ideas, and for the positive vibes when the motivation was fading. My appreciation extends to my co-supervisors. Thank you, **Prof. Dr. Thomas Schmitt**, for all the time taken to try and test things that ended up by not being part of this dissertation, but they were part of the learning process. Thank you, **Dr. Erik Poelman**, for the fruitful conversations through video calls. Your advice and suggestions were priceless.

Thank you, **Thomas Igerst** and **Norbert Schneider**. Your skilful work was invaluable in building the fumigation system. None of my work would have been possible without it. Thank you **Matthias Jäger**, **Beate Krischke** and **Susanne Schiele** for the technical support with all things plants, greenhouse and bees.

Thank you to my many office mates. Thank you, **Benjamin Fuchs**, **Jens Joschinski**, **Julia König**, **Fabian Boetzl**, **Patrick Kohl** and **Justine Vansynghel**. Thank you to my walk and lunch group mates. You have changed throughout the years… but I would particularly like to thank **Fabian Nürnberger**, **Elena Krimmer**, **Friederike Gebert**, **Ludmilla Figueiredo** and **Jie Zhang aka Amy**. Thank you for the lively work-related discussions and for the relaxed non-work-related conversations. They were always a breath of fresh air in the middle of the day.

Thank you, **Jens Rosenberg**, for the encouragement throughout this journey. Thank you for picking up the kid from the kindergarten so that I could work longer days and for taking good care of him in the many weekends I needed to go to work. Thank you for taking care of the little one in order for me to have time to write this thesis. I would not be here without your support.

Funding

I would like to thank for the financial support of the POCH (Operational Human Capital Program, co-financed by the European Social Fund and national funds of the MCTES) through FCT (the Portuguese Foundation for Science and Technology) for her PhD grant (SFRH/BD/110720/2015).

I would also like to thank for the joint financial support given to me at the end of my PhD grant by the Equal Opportunities Commission of the Faculty of Biology and the Department of Animal Ecology and Tropical Biology of the University of Würzburg.

Summary

Anthropogenic activities are causing air pollution. Amongst air pollutants, tropospheric ozone is a major threat to human health and ecosystem functioning. In this dissertation, I present three studies that aimed at increasing our knowledge on how plant exposure to ozone affects its reproduction and its interactions with insect herbivores and pollinators.

For this purpose, a new fumigation system was built and placed in a greenhouse. The annual plant *Sinapis arvensis* (wild mustard) was used as the model plant.

Plants were exposed to either 0 ppb (control) or 120 ppb of ozone, for variable amounts of time and at different points of their life cycle. After fumigation, plants were exposed to herbivores or pollinators in the greenhouse, or to both groups of insects in the field.

My research shows that ozone affected reproductive performance differently, depending on the timing of exposure: plants exposed at earlier ages had their reproductive fitness increased, while plants exposed later in their life cycle showed a tendency for reduced reproductive fitness. Plant phenology was a key factor influencing reproductive fitness: ozone accelerated flowering and increased the number of flowers produced by plants exposed at early ages, while plants exposed to ozone at later ages tended to have fewer flowers. On the other hand, the ozone-mediated changes in plant-insect interactions had little impact on plant reproductive success.

The strongest effect of ozone on plant-pollinator interactions was the change in the number of flower visits received per plant, which was strongly linked to the number of open flowers. This means that, as a rule, exposure of plants to ozone early in the life cycle resulted in a higher number of pollinator visits, while exposure later in the life cycle resulted in fewer flower visits by potential pollinators. An exception was observed: the higher number of visits performed by large syrphid flies to young ozone-exposed plants than to the respective control plants went beyond the increase in the number of open flowers in those plants. Also, honeybees spent more time per flower in plants exposed to ozone than on control plants, while other pollinators spent similar amounts of time in control and ozone-exposed

plants. This guild-dependent preference for ozone-exposed plants may be due to species-specific preferences related to changes in the quality and quantity of floral rewards.

In the field, ozone-exposed plants showed only a tendency for increased colonization by sucking herbivores and slightly more damage by chewing herbivores than control plants. On the other hand, in the greenhouse experiment, *Pieris brassicae* butterflies preferred control plants over ozone-exposed plants as oviposition sites. Eggs laid on ozone-exposed plants took longer to hatch, but the chances of survival were higher. Caterpillars performed better in control plants than in ozone-exposed plants, particularly when the temperature was high.

Most of the described effects were dependent on the duration and timing of the ozone exposure and the observed temperature, with the strongest effects being observed for longer exposures and higher temperatures. Furthermore, the timing of exposure altered the direction of the effects.

The expected climate change provides ideal conditions for further increases in tropospheric ozone concentrations, therefore for stronger effects on plants and plant-insect interactions. Acceleration of flowering caused by plant exposure to ozone may put plant-pollinator interactions at risk by promoting desynchronization between plant and pollinator activities. Reduced performance of caterpillars feeding on ozone-exposed plants may weaken herbivore populations. On the other hand, the increased plant reproduction that results from exposing young plants to ozone may be a source of good news in the field of horticulture, when similar results would be achieved in high-value crops. However, plant response to ozone is highly species-specific. In fact, *Sinapis arvensis* is considered a weed and the advantage conferred by ozone exposure may increase its competitiveness, with negative consequences for crops or plant communities in general. Overall, plant exposure to ozone might constitute a threat for the balance of natural and agro-ecosystems.

Zusammensfassung

Viele anthropogene Aktivitäten verursachen Luftverschmutzung. Unter den Luftschadstoffen stellt das troposphärische Ozon eine Bedrohung für die menschliche Gesundheit und das Funktionieren von Ökosystemen dar. In dieser Dissertation stelle ich drei Studien vor, die darauf abzielen, unser Wissen darüber zu erweitern, wie sich die Exposition von Pflanzen gegenüber Ozon auf ihre Fortpflanzung und ihre Wechselwirkungen mit pflanzenfressenden Insekten und Bestäubern auswirkt.

Zu diesem Zweck wurde eine neue Begasungsanlage gebaut und in einem Gewächshaus aufgestellt. Die einjährige Pflanze *Sinapis arvensis* (Acker-Senf) wurde als Modellpflanze verwendet. Die Pflanzen wurden entweder 0 ppb (Kontrolle) oder 120 ppb Ozon ausgesetzt, und zwar über unterschiedliche Zeiträume und zu verschiedenen Zeitpunkten ihres Lebenszyklus. Nach der Begasung wurden die Pflanzen beider Gruppen im Gewächshaus Pflanzenfressern oder Bestäubern bzw. im Freiland beiden Insektengruppen ausgesetzt.

Meine Forschung zeigt, dass Ozon die Fortpflanzungsleistung je nach Zeitpunkt der Exposition unterschiedlich beeinflusst: Bei Pflanzen, die in einem früheren Alter exponiert wurden, erhöhte sich die Fortpflanzungsfähigkeit, während Pflanzen, die später in ihrem Lebenszyklus exponiert wurden, tendenziell eine geringere Fortpflanzungsfähigkeit aufwiesen. Die Phänologie der Pflanzen war ein Schlüsselfaktor, der sich auf die reproduktive Fitness auswirkte: Ozon beschleunigte die Blüte und erhöhte die Anzahl der Blüten von Pflanzen, die in einem frühen Alter exponiert waren, während Pflanzen, die später exponiert wurden, tendenziell eine geringere Anzahl von Blüten aufwiesen. Andererseits hatten die Veränderungen bei den Interaktionen zwischen Pflanzen und Insekten nur geringe Auswirkungen auf den Reproduktionserfolg der Pflanzen.

Die stärkste Auswirkung von Ozon auf die Interaktionen zwischen Pflanzen und Bestäubern war die Veränderung der Anzahl der Blütenbesuche pro Pflanze, die stark mit der Anzahl der geöffneten Blüten zusammenhing. Dies bedeutet, dass die Exposition von Pflanzen gegenüber Ozon zu Beginn des Lebenszyklus in der Regel zu einer höheren Anzahl von Bestäuberbesuchen führte, während die Exposition zu einem späteren Zeitpunkt des Lebenszyklus zu weniger

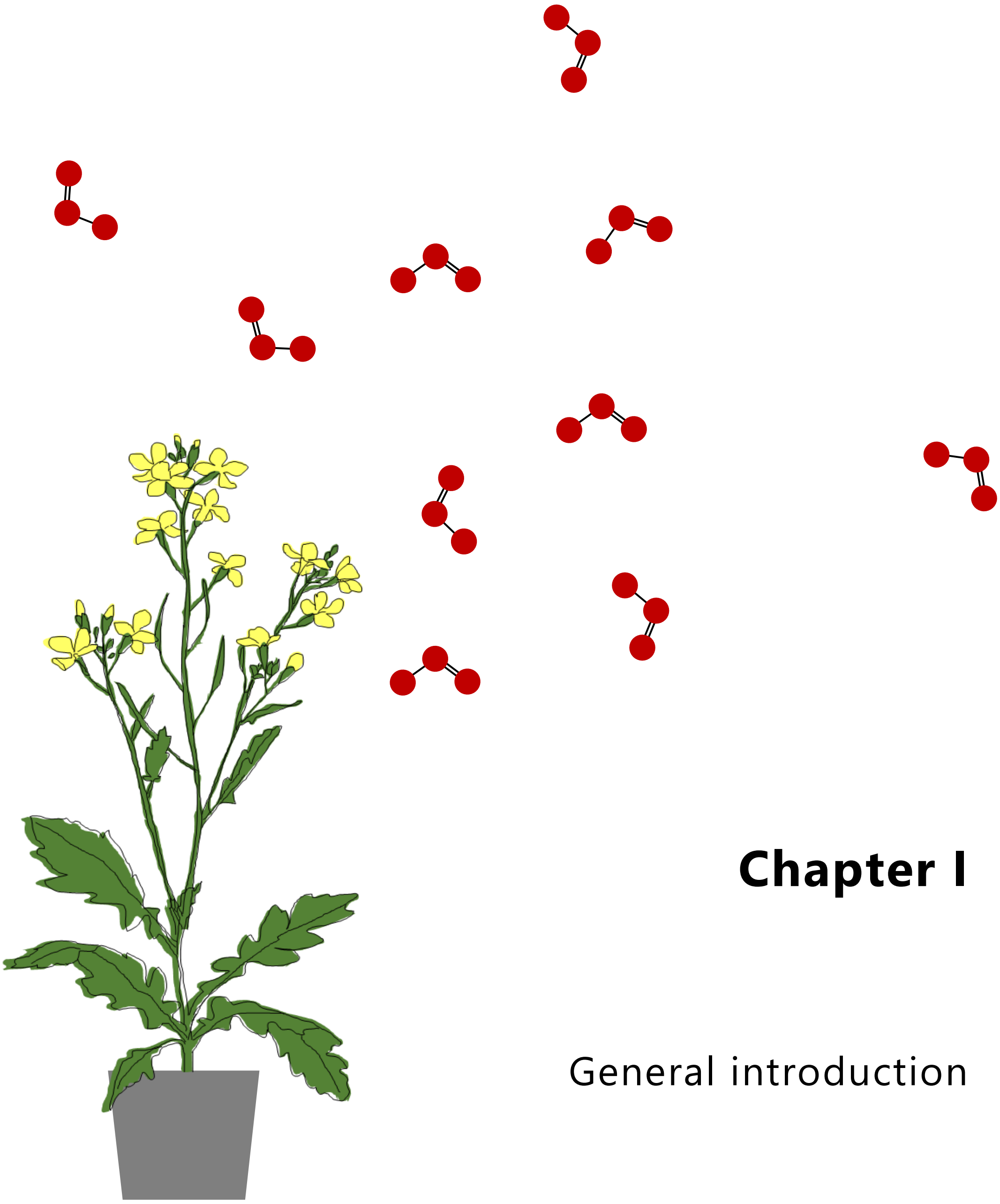
Blütenbesuchen durch potenzielle Bestäuber führte. Eine Ausnahme wurde beobachtet: Die höhere Anzahl der Besuche von großen Syrphiden an jungen, ozonbelasteten Pflanzen im Vergleich zu den entsprechenden Kontrollpflanzen ging über die Zunahme der Anzahl offener Blüten an diesen Pflanzen hinaus. Auch Honigbienen verbrachten mehr Zeit pro Blüte an ozonbelasteten Pflanzen als an Kontrollpflanzen, während andere Bestäuber ähnlich viel Zeit an Kontroll- und ozonbelasteten Pflanzen verbrachten. Diese gildenspezifische Vorliebe für ozonbelastete Pflanzen könnte auf artspezifische Präferenzen zurückzuführen sein, die mit Veränderungen in der Qualität und Quantität der Blütenbelohnung zusammenhängen.

Ozon-exponierte Pflanzen zeigten im Freiland eine tendenziell verstärkte Besiedelung durch saugende Herbivoren und etwas mehr Schäden durch kauende Herbivoren als Kontrollpflanzen. Im Gewächshausversuch hingegen bevorzugten die Schmetterlinge der Art *Pieris brassicae* die Kontrollpflanzen als Eiablageplätze. Die Eier, die auf ozonbelasteten Pflanzen abgelegt wurden, brauchten länger bis zum Schlüpfen, aber die Überlebenschancen waren höher. Die Raupen wachsen auf Kontrollpflanzen besser als auf ozonbelasteten Pflanzen, insbesondere bei hohen Temperaturen.

Die meisten der beschriebenen Effekte hingen von der Dauer und dem Zeitpunkt der Ozonexposition und der beobachteten Temperatur ab, wobei die stärksten Effekte bei längerer Exposition und höheren Temperaturen beobachtet wurden. Außerdem veränderte der Zeitpunkt der Exposition die Richtung der Effekte.

Der erwartete Klimawandel bietet ideale Bedingungen für einen weiteren Anstieg der troposphärischen Ozonkonzentrationen und damit für stärkere Auswirkungen auf Pflanzen und Pflanzen-Insekten-Interaktionen. Die Beschleunigung der Blüte, die durch den Kontakt von Pflanzen mit Ozon verursacht wird, kann die Wechselwirkungen zwischen Pflanzen und Bestäubern gefährden, da sie die Synchronität zwischen den Aktivitäten von Pflanzen und Bestäubern stört. Eine geringere Leistung von Raupen, die sich von ozonbelasteten Pflanzen ernähren, kann die Populationen von Pflanzenfressern schwächen. Andererseits kann die erhöhte Pflanzenreproduktion, die sich aus dem Kontakt junger Pflanzen mit Ozon ergibt, eine gute Nachricht für den Gartenbau sein, wenn ähnliche Ergebnisse bei hochwertigen Nutzpflanzen erzielt werden. Die Reaktion der

Pflanzen auf Ozon ist jedoch sehr artspezifisch. Sinapis arvensis gilt als Unkraut, und der Vorteil, der sich aus der Ozonexposition ergibt, könnte seine Wettbewerbsfähigkeit erhöhen, was negative Folgen für die Kulturpflanzen oder Pflanzengemeinschaften im Allgemeinen hätte. Insgesamt könnte die Exposition von Pflanzen gegenüber Ozon eine Bedrohung für das Gleichgewicht von natürlichen und landwirtschaftlichen Ökosystemen darstellen.



Chapter I

General introduction

Plant-insect interactions are of major importance for the balance of natural and agroecosystems. Herbivory and pollination are two types of interactions that affect plant fitness and ultimately plant persistence. However, anthropogenic activities are reducing insect abundance and threatening these interactions. Air pollution is a consequence of anthropogenic activities. In particular, tropospheric ozone is a highly oxidative gaseous pollutant with a great potential to affect plant-insect interactions. Furthermore, the levels of ozone in the troposphere suffered a tremendous increase compared with pre-industrial times and, globally, ozone levels are still expected to continue increasing in the coming years. The objective of my research was to bring further enlightenment to the body of knowledge on the plant-mediated effects of ozone on plant-insect interactions and the consequences for plant reproduction. Having this purpose in mind, a new fumigation system was developed for exposing plants to different levels of ozone. Experiments were then carried out to study: a) how the duration of plant exposure to ozone affects the life cycle of an herbivore (chapter II); b) how plant exposure to ozone affects pollination by four selected pollinator species (chapter III); and c) how the plant age at the time of exposure to ozone affects the plant interactions with herbivores and pollinators in the field (chapter IV).

Trends in tropospheric ozone levels

In the dawn of atmospheric ozone research, tropospheric ozone was believed to originate in the stratosphere, in the so-called ozone-layer. Nowadays, we know that, although stratospheric ozone provides a contribution, most of the ozone that we breathe is a result of anthropogenic activities (Young et al., 2013). Ozone is produced by photochemical reactions between primary pollutants, namely nitrogen oxides and volatile organic compounds (VOCs) (Pinto et al., 2010). Since the industrial revolution, pollutant emissions have led to a 40 % increase in tropospheric ozone levels (Yeung et al., 2019). Currently, due to pollutant emission regulations (EPA, 1990; EU, 2008; The Chinese State Council, 2013), many parts of the world are witnessing a reduction in tropospheric ozone concentrations (Lin et al., 2017; Yan et al., 2019). However, modelling approaches continue to predict an increase in the global average ozone concentration (Fuhrer et al., 2016) and in the number of ozone episodes (Lei et al., 2012) in the near future.

Vegetation can act both as source and sink for ozone: it contributes to the formation of ozone through the production of biogenic VOCs, but it also contributes to its removal through dry deposition on leaf surfaces or stomatal uptake (Fitzky et al., 2019). In the so-called ozone-climate penalty, the predicted climate change (increased average air temperature, increased frequency of heatwaves and droughts) may override, or at least reduce, the decrease in ozone formation resulting from the reduction of anthropogenic precursor emissions. These referred conditions are not only ideal for the formation of ozone, but also for its longer persistence in the troposphere, through the reduction of ozone removal by water-stressed vegetation (Lin et al., 2020). Nitrogen oxides are often transported from urban to rural areas. Here, the VOCs needed for ozone production are supplied by the vegetation and are not a limiting factor, making ozone concentrations typically higher in rural areas (Fitzky et al., 2019; Yan et al., 2018), although these are also the areas where most of the benefits of the reduced anthropogenic emissions on ozone concentrations are being observed (Fitzky et al., 2019; Yan et al., 2019). High concentrations of ozone in rural areas are a threaten to natural and cultivated plants.

Ozone and plant reproduction

Exposure to ozone and ozone uptake can be highly detrimental to plants with consequences to their reproduction (Emberson et al., 2018). In the year 2000, ozone was responsible for global economic losses of 11 – 18 billion dollars due to decreased yields of wheat, soybean and maize (Avnery et al., 2011a). However, negative effects on reproduction are not exclusively observed in crops (Leisner and Ainsworth, 2012). Therefore, ozone constitutes a threat both to food security and the balance of ecosystems.

Some of the mechanisms through which ozone directly affects plants include reduction of photosynthetically active area through chlorotic/necrotic spots, decreased Rubisco activity (Fiscus et al., 2005) and alteration of pollen and ovule viability (Gillespie et al., 2015; Leisner and Ainsworth, 2012)

Ozone and plant-insect interactions

An indirect way that ozone might affect plants is by changing their interactions with insects. Whether they are antagonistic, such as herbivory, or mutualistic, as is the case of pollination, plant interactions with insects play an important role in plant fitness and, therefore, in shaping plant communities. Herbivores consume 18 % of the aboveground terrestrial plant biomass (Cyr and Face, 1993), reducing the foliar area available for photosynthesis and the potential reproductive sites, which reduces plant fitness and reproductive performance (Crawley, 1989). On the other hand, pollinators are involved in the sexual reproduction of 88 % of the world’s angiosperms (Ollerton et al., 2011). Moreover, it was estimated that, in 2005, the economic value of the pollination services was 39 % of the global value of food crops (Gallai et al., 2009). It is therefore imperative to protect these interactions.

Many insects depend on the odours emitted by plants to recognize their hosts or food sources, often relying on very specific odour blends (Bruce and Pickett, 2011; Schiestl, 2015). Tropospheric ozone has the potential of changing the perception of VOCs in several ways. Ozone stress may lead plants to emit a different blend of volatiles (Saunier and Blande, 2019). Emitted VOCs may react with ozone in the atmosphere, modifying the odour blends (Li et al., 2016). Insects may have their perception impaired by contact of ozone with their antenna (Démares et al., 2022; Dotterl et al., 2016; Vanderplanck et al., 2021). There is also the possibility that ozone affects the visual cues provided by the plants, namely by altering flower colour and flower display (Prieto-Benitez et al., 2021).

Factors influencing the effects of ozone

Many factors may influence how ozone exposure affects plants and their interactions with insects. The most obvious one is the dose of exposure. Ozone exposure may be classified as either chronic (exposure to relatively low concentrations for longer periods) or acute (exposure to high concentrations of ozone for short periods). Therefore, the dose of exposure is characterised by the ozone concentration and the duration of the exposure. While ozone concentration is an important factor considered in most studies, the duration of the exposure has not been the focus of former studies.

Another set of factors to be considered are climatic conditions such as temperature, relative humidity and water pressure deficit, that may influence the extent of the effects of ozone. Changes in these conditions alter the susceptibility of the plants to ozone, because they change stomatal conductance and, consequently, ozone uptake (Emberson et al., 2018).

Plant sensitivity to abiotic stress varies as plants age (Rankenberg et al., 2021). However, the effect of plant age at the time of exposure has rarely been considered for ozone stress.

Research questions

In my doctoral research I used the annual plant *Sinapis arvensis*, or wild mustard, as the model plant. This plant was chosen on the basis of being self-incompatible and highly dependent on insect pollinators (Mulligan and Bailey, 1975).

In a set of three studies, I tried to answer the following research questions.

1) Is plant reproductive performance affected when the plant is exposed to enhanced levels of ozone?

When the answer to this question would be "yes", several questions would follow:

2) Is this due to changes in the flowering patterns of the plant?
3) Is this due to changes in the vegetative development of the plant?
4) Is this due to changes in plant-insect interactions?

On the plant-mediated effects of ozone on plant-insect interactions, I asked:

5) Is the life cycle of the specialist insect-herbivore *Pieris brassicae* affected by plant exposure to ozone?
    a) Does plant exposure to ozone affect the oviposition preference of a butterfly?
    b) Does plant exposure to ozone affect the duration of the egg phase?
    c) Does plant exposure to ozone affect the egg survival rate?
    d) Does plant exposure to ozone affect the performance of the caterpillars?

6) Is pollinator attraction affected by plant exposure to ozone?
    a) Is the number of flower visits affected by plant exposure to ozone?
    b) Is the duration of pollinator visits affected by exposure to ozone?
    c) Do different pollinator species respond differently to plants exposed to ozone?

I further investigated if there were other factors influencing the effects of ozone:

7) Are the effects dependent on the duration of the exposure?
8) Are the effects dependent on the plant age at the time of exposure?
9) Are the effects dependent on climatic conditions?

The fumigation system

To answer the previous questions, a new fumigation system was developed to expose plants to ozone. I aimed at building a system where one could grow and fumigate plants, with the additional possibility of collecting plant volatile organic compounds directly from the growing/fumigation chambers. With the idea of using as much natural light as possible for growing the plants, the main building material of the chambers is glass. Besides glass, preference was given to materials that are also inert and do not emit their own volatiles like stainless steel and Teflon.

The system includes two glass chambers, with a capacity of about 1000 L each (Fig. I.1). A stream of compressed air creates the necessary airflow into the chambers. This airflow is cleaned by moving through an activated charcoal filter followed by a particle filter. The concentration of ozone in the resulting air is approximately 0 ppb.

A portion of the cleaned air flows through an air dryer (AIRdryer3.1, INNOTEC) followed by a customized ozone generator (INNOTEC high engineering GmbH) where an adjustable fraction of the oxygen will be converted into ozone. This portion of the air is then added to the main stream of the incoming air of one of the chambers, enhancing the ozone concentration. The ozone concentration in that chamber is continuously monitored by an ozone analyser (APOA-370, Horiba Ltd), whose information is used by a controller that feeds back to the ozone generator, with the purpose of achieving the desired ozone concentration in the chamber. A timer allows to control the fumigation schedule and two rotameters level the amount of incoming air in the two chambers.

Methods

In the experiments reported in this dissertation, *Sinapis arvensis* plants were exposed to two different concentrations of ozone in the fumigation chambers. Afterwards, interactions between the plants and their herbivores and pollinators were assessed.

Plant fumigation

The *Sinapis arvensis* seeds used in my research came from a population maintained by the botanical garden of Konstanz. Seeds were sown in trays and each plant was repotted into an 18 x 18 cm pot after 2 weeks.

In chapter III, I tested whether the effects of ozone depend on the age of the plant at the time of ozone exposure. For that purpose, plants were exposed when

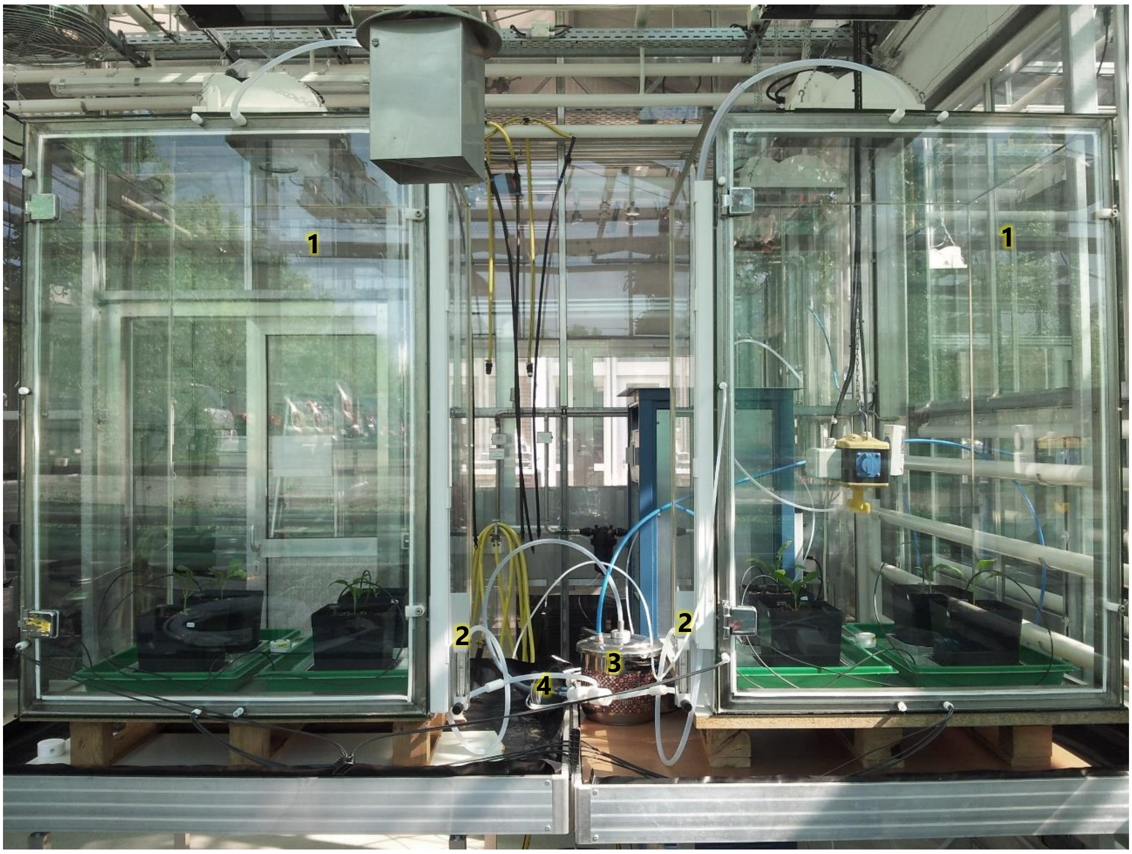


Figure I.1 – The fumigation system. A) The glass chambers. 1 – Glass chambers; 2 – Flowmeters; 3 – Activated charcoal filter; 4 – Particle filter. B) The electronic devices. 5 – Ozone analyzer; 6 – Ozone generator; 7 – Timer; 8 – Controller; 9 – Air dryer.

they were 3, 4, 5 or 6 weeks old. In the remaining experiments, plants were exposed to ozone four weeks after sowing. Fumigation consisted of exposing the plants either to 0 or 120 ppb of ozone for 6 hours per day, between 11h00 and 17h00 CET. The treatment level in the two fumigation chambers was assigned randomly. The ozone concentration outside the fumigation period was ~ 0 ppb in both chambers. In chapter II, duration of exposure was considered as a potential factor influencing the effects of ozone and plants were exposed for either 1 day or 5 consecutive days. In chapters III and IV, plants were exposed for 5 and 7 days, respectively. This is considered an acute exposure. Tropospheric ozone concentrations of 120 ppb of ozone are very high, but it is not unusual for these concentrations to be exceeded (Lei et al., 2012).

Plant-insect interactions

After exposure in the fumigation chambers, plants were presented to an herbivore (chapter II) or to four selected pollinators (chapter III) in the greenhouse, or presented to all insects in the experimental garden (chapter IV).

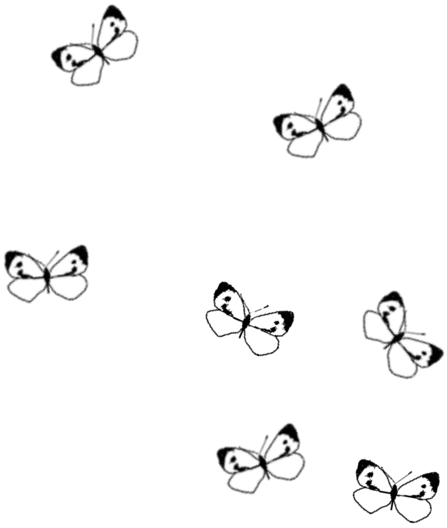
In chapter II, the oviposition preference of the specialist herbivore *Pieris brassicae* and the performance of its brood were assessed. Temperature was considered as a factor influencing the effects of ozone.

In chapter III, flower visits by the solitary bee *Osmia bicornis*, the social bees *Apis mellifera* and *Bombus terrestris* and the syrphid fly *Eristalis tenax* were investigated.

In chapter IV, fumigated plants were openly presented to all insects. Herbivore presence and damage were recorded. Flower visitation by potential pollinators was assessed.

Plant performance

At fruit maturity, plant reproductive performance was estimated. All fruits were counted and a portion of the fruits were assessed for the number of seeds and the seed weight (chapter III and IV). Also at fruit maturity, the weight of the vegetative parts was measured (chapter III).



Chapter II

Plant-mediated effects of ozone on herbivores depend on exposure duration and temperature

Abiotic stress by elevated tropospheric ozone and temperature can alter plants' metabolism, growth, and nutritional value and modify the life cycle of their herbivores. We investigated how the duration of exposure of Sinapis arvensis plants to high ozone and temperature levels affect the life cycle of the large cabbage white, Pieris brassicae. Plants were exposed to ozone-clean (control) or ozone-enriched conditions (120 ppb) for either 1 or 5 days and were afterwards kept in a greenhouse with variable temperature conditions. When given the choice, P. brassicae butterflies laid 49% less eggs on ozone-exposed than on control plants when the exposure lasted for 5 days, but showed no preference when exposure lasted for 1 day. The caterpillars took longer to hatch on ozone-exposed plants and at lower ambient temperatures. The ozone treatment had a positive effect on the survival of the eggs. Ozone decreased the growth of caterpillars reared at higher temperatures on plants exposed for 5 days, but not on plants exposed for 1 day. Overall, longer exposure of the plants to ozone and higher temperatures affected the life cycle of the herbivore more strongly. With global warming, the indirect impacts of ozone on herbivores are likely to become more common.

This chapter is published as **Duque, L.**, Poelman, E.H., Steffan-Dewenter, I., **2019**. *Plant-mediated effects of ozone on herbivores depend on exposure duration and temperature*. Scientific Reports 9, 19891.

Introduction

Ozone is a highly oxidative gas with widely recognized detrimental effects on human health (Nuvolone et al., 2018) and plant growth (Ashmore, 2005). Far less documented are the effects of ozone on plant-animal interactions, but see (Abu ElEla et al., 2018; Agathokleous et al., 2017; Couture and Lindroth, 2012; Couture et al., 2012; Cui et al., 2014; Farre-Armengol et al., 2016; Fuentes et al., 2013; Khaling et al., 2016; Khaling et al., 2015; Peltonen et al., 2006; Peltonen et al., 2010; Pinto et al., 2010) for some examples.

Tropospheric ozone is a secondary gaseous pollutant that forms through photochemical reactions of its precursors (nitrogen oxides and volatile organic compounds) (Pinto et al., 2010). Despite the efforts to reduce precursor emissions in some parts of the world, there is a high probability that, globally, the average concentration of tropospheric ozone is still going to increase in the coming years (Fuhrer et al., 2016). Rising precursor emissions in rapidly growing economies, mainly in Asia and global warming are expected to be the source of the problem (Fuhrer et al., 2016). Also, controlling precursor emissions locally is not as effective in reducing ozone concentrations as desired due to long-range transport of pollution (Lin et al., 2017). Furthermore, simulations for the year 2050 point to increased frequency of high ozone episodes in developing regions in the best-case scenario, but to a generalized increase in ozone episodes when the use of fossil fuels keeps increasing (Lei et al., 2012).

Tropospheric ozone is reported to affect plant photosynthesis, with consequences for plant growth, plants' nutritional value and crop yield (Emberson et al., 2018). Many cases of visible plant injury and biomass reduction due to ozone exposure have been reported in Europe, with 39% of these happening in crops (Mills et al., 2011). The estimated global economic losses deriving from yield losses of wheat, soybean and maize for the year 2000 ranged between $11 and 18 billion (Avnery et al., 2011a) and are expected to increase by $1 to 17 billion in 2030, depending on the metrics used and the emission scenarios considered (Avnery et al., 2011b).

By changing the plants' metabolism, ozone may indirectly affect the interaction between plants and other organisms through quantitative or qualitative changes in the production of plant secondary metabolites involved in the communication between plants or in plant attraction/repellence/defence (Cui et al., 2014; Himanen et al., 2009; Khaling et al., 2016). Ozone may also react with volatile secondary metabolites in the atmosphere, breaking them down into unknown reaction products, possibly disrupting the communication with other organisms (Fuentes et al., 2013; Giron-Calva et al., 2016; Li et al., 2016; McFrederick et al., 2009). Furthermore, ozone may change the nutritional value or the toxicity of the plants, altering the performance of herbivores feeding on these plants (Couture and Lindroth, 2012; Couture et al., 2012; Holton et al., 2003; Jackson et al., 2000; Khaling et al., 2015; Peltonen et al., 2010).

In this study, we focus on the interaction between the herbivore *Pieris brassicae* L. (large cabbage white) and the host plant *Sinapis arvensis* L. (wild mustard). *P. brassicae* is a butterfly whose caterpillars are specialist herbivores feeding on Brassicaceae plants that contain glucosinolates (Smallegange et al., 2007). Wild mustard, *S. arvensis* is one of those plants. It is indigenous to Europe, the Middle East and Western Asia and is nowadays an important weed of field crops in most of the temperate regions of the world (Warwick et al., 2000).

The interaction between *S. arvensis* and *P. brassicae* starts at the point when a female butterfly searches for and chooses a suitable place for laying her eggs. This process is usually guided by chemical cues produced by the plants (Hilker and Meiners, 2011). Once a choice is made, the butterfly usually lays its eggs in clusters attached to the under surface of leaves (David and Gardiner, 2009). After hatching, the insects use the plant as food source. From the plants perspective, the larval stage of the herbivore is an antagonist, and may induce a defensive response, both at egg and caterpillar stage. Plant defence mechanisms can be induced by egg deposition or caterpillar damage and include a hypersensitive response-like necrosis that kills the eggs by desiccation (direct defence), the production of toxins (direct defence) or the production of chemical cues for recruiting parasitoids (indirect defence) (Dicke et al., 2009; Fatouros et al., 2016; Pashalidou et al., 2015b).

Plant stressors, such as ozone, may affect each of these interactions. Studies on the effects of ozone on the oviposition preference of insects revealed that exposure of host plants to ozone had positive (Jackson et al., 1999), negative (Cui et al., 2014; Jones and Coleman, 1988; Kopper and Lindroth, 2003) or no effects (Peltonen et al., 2006) on the oviposition preference of the insects. Furthermore, one study showed that ozone has an effect on the fecundity of the females (Couture and Lindroth, 2012). Also, several studies have reported a plant-mediated effect of ozone on caterpillar development, be it a positive (Bolsinger et al., 1992; Jackson et al., 2000) or a negative one (Jondrup et al., 2002; Khaling et al., 2015). In some cases, previous exposure of the plant to ozone altered the consumption of plant material (Jones and Coleman, 1988; Khaling et al., 2015).

Factors that may explain contradicting findings are the ozone exposure level and interactive effects with temperature. Ozone exposure is defined by the concentration of ozone, the frequency and the duration of exposure. Previous studies on the effects of ozone on plant-insect interactions considered acute exposure (Jones and Coleman, 1988; Khaling et al., 2015) (for a short period but with very high ozone concentrations) or chronic exposure (Abu ElEla et al., 2018; Agathokleous et al., 2017; Couture and Lindroth, 2012; Couture et al., 2012; Peltonen et al., 2006) (often covering most of the life span of the plant, with concentrations that usually do not exceed 2 times the ambient ozone concentrations). However, the duration of exposure is not considered as a factor in these studies. Therefore, it is uncertain, particularly for acute exposures, whether the effects of ozone would be additive or there would be recovery and a compensatory mechanism after an initial response.

Furthermore, little is known about the interactive effects of elevated ozone and temperature (Mills et al., 2016). Although elevated temperature increases plant growth, the combined effects of temperature and ozone on plants seem to be case-specific (Kasurinen et al., 2012). Elevated temperature also increases insect metabolism (Jaworski and Hilszczański, 2013), affecting plant-insect interactions through changes in consumption (Lemoine et al., 2014). Studies that integrate the effects of both temperature and ozone on plant-insect interactions are missing, although higher mean temperatures under future climatic conditions may reinforce or attenuate the effects of ozone on these interactions.

In this study we performed a greenhouse experiment to assess the response of *Pieris brassicae* to *Sinapis arvensis* plants exposed to 2 levels of ozone (0 and 120 ppb) for 2 different periods (1 and 5 days). Specifically, we address the following questions: 1) Does a previous exposure of plants to ozone affect: a) the oviposition preference of the butterfly?; b) the duration of the egg stage?; c) the egg survival rate?; d) the caterpillar performance? 2) Does the duration of exposure influence the way ozone affects these parameters of the herbivore life cycle? 3) How is temperature modulating the way ozone affects these parameters?

Material and methods

Biological material

Wild-type *Sinapis arvensis* seeds were provided by the Botanical Garden of Konstanz, Germany. Wild mustard plants were grown in 18 x 18 cm pots in a 2:1-mixture of peat based substrate (Einheits Erde CL ED 73) and sand (Hamann Filtersand 0,7-1,25 mm). The plants grew in a greenhouse in the Biocenter of the University of Würzburg under 50%/80% relative humidity and 16h/8h, dark/light respectively. To keep the photoperiod constant, supplementary illumination was applied by high-pressure sodium lamps whenever the light intensity outside the greenhouse would drop below 20 kLux. Although this greenhouse can be heated to increase the temperature, the aeration system does not allow to decrease the temperature in warm, sunny days. During this experiment, the temperature was recorded every 12 minutes and later included as a potential explanatory variable. The overall temperatures in the greenhouse ranged between 14.0 and 35.1 °C.

Pieris brassicae butterflies were obtained from a population that is routinely reared on *Brassica oleracea* var. *gemmifera* cultivar Cyrus in the Laboratory of Entomology at Wageningen University, The Netherlands. As the experiment continued, we also used butterflies reared in our greenhouse, descendants from the same population. The butterflies were kept in the greenhouse, inside a 1.15 m-high insect rearing tent (Bugdorm) and were fed a 10-20% honey solution.

Ozone exposure system

In the greenhouse, we installed an ozone-exposure system (Fig. II.1) consisting of: 1) 2 glass chambers with a stainless steel door frame and an approximate volume of 1000 L each; 2) a customized ozone generator (INNOTEC high engineering GmbH) linked to an air dryer (AIRdyer3.1, INNOTEC) ; 3) an ozone analyser (APOA-370, Horiba Ltd); 4) a controller that links the ozone analyser and the ozone generator, allowing us to regulate the ozone concentration in the chamber; 5) a timer; 6) a main air stream of compressed air passing through an activated charcoal filter and a particle filter before it reaches the chambers; 7) a secondary air stream, branching from the main one, passes through the air dryer and the ozone generator and disembogues in the main air stream, allowing for ozone enrichment of the incoming air; 8) 2 rotameters allow us to level the amount of incoming air to each chamber to 70 L/min. Because the chambers are almost exclusively made of glass, the photoperiod conditions are the same as in the greenhouse itself.

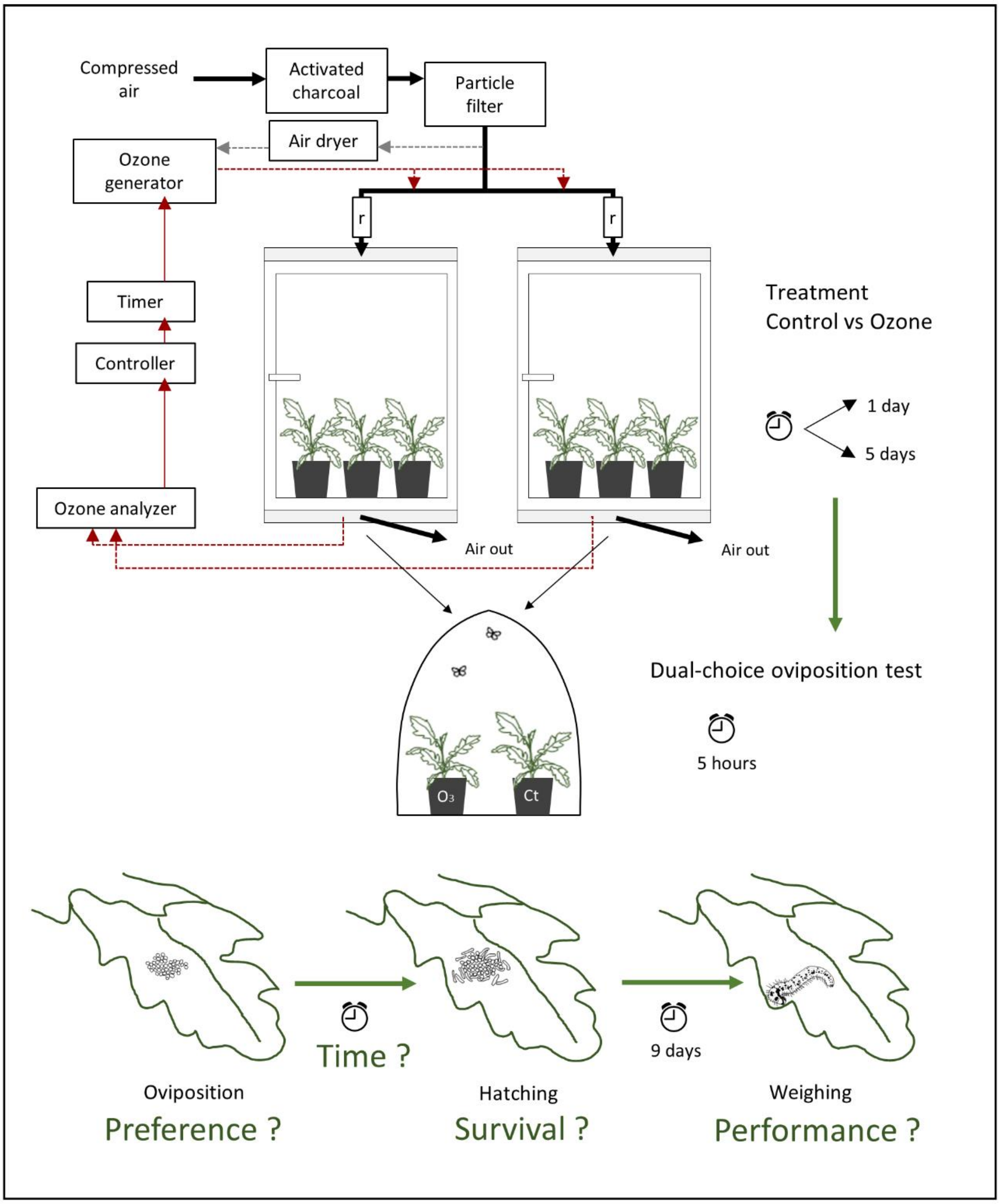


Figure II.1 – Representation of the ozone-exposure system, the experiment and the main research questions. r - rotameter.

The two chambers allow to have, at any given time, an ozone-clean environment and an ozone-enriched environment, and the treatments are interchangeable. The ozone analyser is constantly sucking in the air from one of the chambers, analysing it for its ozone content. This information is then passed on to the controller that gives feedback to the ozone generator, switching it on or off in order to achieve the desired average ozone concentration, which can be modified in the controller. Pre-experiment tests revealed that the ozone concentration of the clean air chamber ranges between 0 and 1 ppb. Therefore, the ozone concentration is only monitored in the ozone-enriched chamber.

Study design

This study was performed between February and May 2018. The plants were fumigated before being presented to the herbivores. The fumigation treatments consisted of exposing the plants to 2 levels of ozone (control vs ozone) for 2 different periods (1 or 5 days). In the control treatment plants were exposed to clean air (~0 ppb ozone) for the entire day, whilst in the ozone treatment, the plants were exposed to elevated-ozone conditions (~120 ppb ozone, Table SII.1) for 6 hours/day, between 11h00 and 17h00, and to clean air for the rest of the day. 240 $\mu g.m^{-3}$ (~120 ppb) is the alert threshold for ozone established by the European Union. In Europe, exceedances of this value occur usually in summer and, although uncommon, several days with exceedances may be observed in the period of one year(EEA, 2006, 2010, 2014).

At the start of each experimental round, the duration of the exposure was fixed and each fumigation chamber was randomly assigned a treatment (control vs ozone). Four weeks after sowing, eight plants were placed in each chamber and exposed to the corresponding treatment.

Following ozone exposure, one plant from each treatment was enclosed in an insect-rearing tent along with one female and one male *P. brassicae* butterfly. The butterflies remained in the tents for 5 hours, after which the number of eggs laid on each of the two plants were counted and the plants were measured. The health status of the plant was recorded: percentage of leaves showing signals of visible ozone damage (chlorosis/necrosis) and percentage of leaves infected by powdery mildew, a fungal infection. Each butterfly was only used for one oviposition test.

Plant pairs that were offered to butterflies that did not lay any eggs during the 5-hour oviposition period were excluded from the oviposition preference analysis.

After the oviposition preference tests, all plants were kept in a greenhouse compartment on plant saucers surrounded by water to avoid hatched caterpillars to migrate to a different plant. The plants were monitored daily for detection of caterpillar hatching.

The duration of the egg phase was calculated for the eggs laid on each plant, as the number of days between the day of the oviposition tests and the day when the majority of the caterpillars hatched. When none of the eggs laid by a butterfly hatched, we considered that it was likely that they were not fertile and they were excluded from the analysis of egg survival rate. This was the case for 4 control and 3 ozone replicates for 1-day exposed plants and 2 control and 2 ozone replicates for 5-day exposed plants.

After hatching, all caterpillars were retrieved from the plants and counted. Afterwards, 10 caterpillars were reintroduced on the plants where they had hatched. Plants that had no eggs or that had eggs that did not hatch also received 10 caterpillars from plants of the same treatment. In both cases the newly hatched caterpillars were introduced on the fourth oldest leaf. Nine days after the introduction of the caterpillars, they were removed from the plants and weighed.

In total, we performed 13 experimental rounds: 6 for the 1-day treatment and 7 for the 5-day treatment. One of the 1-day treatment rounds was not completed since the caterpillars did not hatch, presumably due to desiccation and, therefore, we had no larvae to reintroduce.

Statistical analysis

All statistical analyses were performed using R (R Core Team, 2017) (version 3.4.2). The data collected for the 1-day and 5-day exposed plants were analysed separately, with test results and model fitting presented separately for each duration of treatment.

A binomial test to compare proportions of injured plants was performed to evaluate the effect of the ozone treatment on plant injury (prop.test function).

We used the dredge function (MuMIn package (Barton, 2018), version 1.42.1) to assess which models best explain our data. The equations in this section show the variables that were considered as potential predictors in the global models.

For the oviposition preference tests, we used the number of eggs laid on the plants as the response variable. We fitted a generalized mixed effects model for zero-inflated data with butterfly as the random factor (glmmTMB, glmmTMB package (Brooks et al., 2017), version 0.2.2.0, family=negative binomial).

*Number of eggs laid ~ Treatment * fungal infection + Plant height*

To check if the tendency observed on the number of eggs laid per plant was kept after hatching, we refitted the generalized mixed effects model with zero-inflated data and butterfly as the random factor for the number of caterpillars hatched per plant (glmmTMB, family=negative binomial).

*Number of caterpillars per plant ~ Treatment * fungal infection*

We used a generalized mixed effects model with butterfly as a random factor to analyse the egg survival rate (glmer, lme4 package (Bates et al., 2015), family=binomial, link=logit).

*Egg survival ~ Treatment * Average temperature during the egg stage + Treatment * Number of eggs per plant*

The duration of the egg stage was analysed, by fitting a linear mixed effects model with butterfly as the random factor (lmer, lme4 package (Bates et al., 2015), version 1.1-17).

*Duration of egg stage ~ Treatment * Average temperature during the egg stage + Treatment * Number of eggs per plant*

Fungal infection was not included in the global models to analyse the duration of the egg stage and the egg survival because infection rate was highly correlated with the average temperature during the egg stage and the latter revealed a stronger predictor for both variables than fungal infection. We took butterfly as a random factor in the previous models, to account for 1) the dependency in the number of eggs laid on the two plants exposed to the same butterfly, 2) the fact that eggs laid by the same butterfly are expected to have similar quality, leading to similar egg survival and similar duration of the egg stage.

The weight of the caterpillars was cube root-transformed in order to approximate a normal distribution and was analysed with a linear mixed effect model (lmer) with the plant as random factor.

*Caterpillar weight ~ Treatment * number of laid eggs + Treatment * Average temperature during the caterpillar stage + Treatment * fungal infection*

The number of eggs was included as a potential predictor for caterpillar weight, because egg deposition was shown to affect the development of caterpillars in several species of Brassicaceae (Pashalidou et al., 2015a).

Model validation was performed by visually checking the residuals for the linear models and by simulating residuals with the Dharma package (Hartig, 2018) (version 0.2.0) for the generalized linear models.

In the results' section we present the models with the lowest AICc for each research question. Alternative models (ΔAICc < 2.0) are shown in the supplementary material (Tables SII.2-SII.6). The number of replicates for each research question is provided in Table SII.7.

Results

We observed signs of plant injury on plants exposed to ozone. While 2% of the plants exposed to ozone for 1 day and 39% of the plants exposed to ozone for 5 days showed chlorosis/necrosis in several degrees, none of the control plants showed visible injury. This indicates a strong effect of ozone on plant injury for a 5-day long treatment ($\chi^2 = 24.945$, df = 1, $p < 0.001$), but not for a 1-day long treatment.

During the 5-hour periods of the oviposition dual-choice assays, each female butterfly laid between 1 and 233 eggs, with a mean of 96 eggs. When given the choice between a plant previously exposed to ozone and a control plant, butterflies chose to either lay all the eggs on one of the plants or distribute them between the two plants they were offered. The butterflies laid 15% less eggs on plants exposed to ozone for 1 day and 49% less eggs on plants exposed to ozone for 5 days than on the respective control plants (Fig. II.2A). The best model based on

AICc (Table II.1) indicates an effect of ozone on the oviposition preference of the butterfly when the plants were exposed for 5 days (z=-1.830 p=0.067), but not when the plants were exposed for only one day (Fig. II.2A, Table II.1). The health status of the plant also affected the number of eggs laid, with fewer eggs being laid on plants with a higher fungal infection rate (Table II.1, z=-2.346, p=0.019 and z=-2.088, p=0.037, for 1- and 5-day exposed plants respectively).

Table II.1 – Summary of the models with the lowest AICc for each response variable.

	Response variable									
Predictors	Number of laid eggs		Number of caterpillars on plants		Egg survival		Duration of egg phase		Larval weight	
1day	z	P	z	P	z	P	t	P	t	P
Treatment	------	------	------	------	2.962	**0.003**	2.127	**0.040**	------	------
Fungal infection	-2.346	**0.019**	-2.248	**0.027**					-------	------
Plant height	------	------								
Temp egg					1.923	**0.055**	-17.27	**<0.001**		
Temp larva									22.950	**<0.001**
Number of eggs					1.885	**0.059**			------	------
Treat x Fung inf	------	------	------	------					------	------
Treat x temp egg					-2.796	**0.005**	------	------		
Treat x temp larv									------	------
Treat x nr eggs					------	------	------	------	------	------
R²					0.62		0.88		0.89	
5 days	z	P	z	P	z	P	t	P	t	P
Treatment	-1.830	**0.067**	-2.209	**0.027**	2.352	**0.019**	2.017	**0.062**	2.76	**0.007**
Fungal infection	-2.088	**0.037**	------	------					-3.141	**0.002**
Plant height	------	------								
Temp egg					------	------	-4.157	**<0.001**		
Temp larva									12.102	**<0.001**
Number of eggs					2.633	**0.008**			------	------
Treat x Fung inf	------	------	------	------					------	------
Treat x temp egg					------	------	------	------		
Treat x temp larv									-2.856	**0.005**
Treat x nr eggs					------	------	------	------	------	------
R²					0.74		0.90		0.88	

Treat = Treatment (Control vs Ozone); Fung inf = Fungal infection (percentage of leaves infected by powdery mildew); Temp egg = average ambient temperature during the egg stage; Temp cat = average ambient temperature during the caterpillar stage. --------- indicates variables that were considered for the best model but were not included in the model with the lowest AICc. Empty cells indicate variables that were not considered for the best model. We present t or z-values, depending if the fitted model is a linear mixed effects model or a generalized linear mixed effects model, respectively.

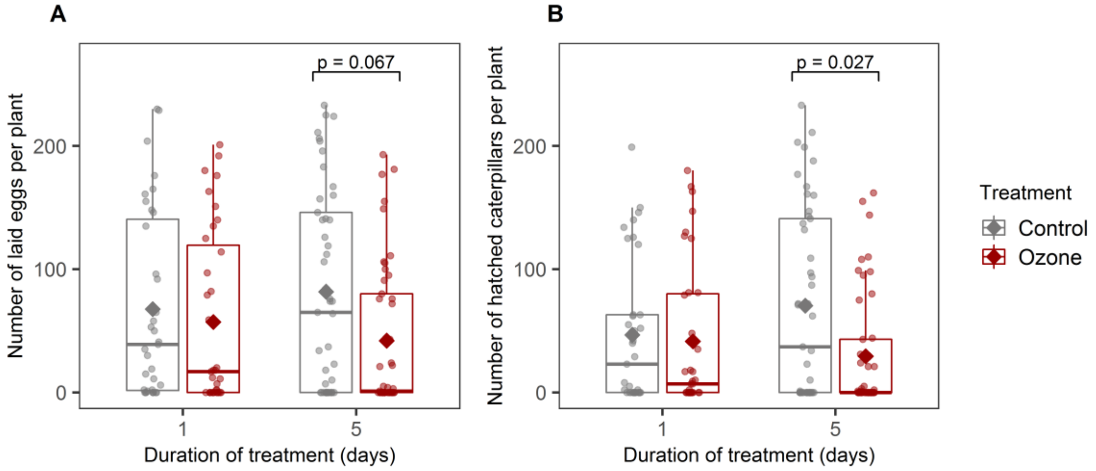


Figure II.2 - Plant-mediated effects of 2 levels of ozone exposure (1 and 5 days) on (A) the number of eggs laid by *Pieris brassicae* butterflies on dual-choice assays and (B) the number of caterpillars per plant after hatching. The dots represent the data points, the diamonds correspond to the means. P-values for the effect of the treatment, given by the reported models, are shown.

After hatching, the number of caterpillars was 11% and 58% lower on ozone-exposed plants than on control plants, for 1- and 5-day exposed plants respectively. However, the effect of ozone was only significant for the longer exposure (z=-2.209, p=0.027; Fig. II.2B).

The ozone treatment had a positive effect on the survival of the eggs (z=2.962, p=0.003 and z=2.352, p=0.019, for 1- and 5-day exposed plants respectively, Fig. II.3A and II.3B). The survival was also positively affected by the number of eggs laid per plant (z=1.885, p=0.059 and z=2.633, p=0.008, for 1- and 5-day exposed plants respectively, Fig. II.3A and II.3B).

The ambient temperature during the egg stage had a positive effect on the survival of eggs laid on 1-day exposed plants (z=1.923, p=0.055). A significant interaction between the treatment and the temperature during the egg stage (z=-2.796, p=0.005), revealed that the temperature only affected the survival of the eggs laid on control plants (Fig. SII.1).

The eggs took between 4 and 8 days to hatch (mean 5.8 days) and this was highly dependent on the temperature during the egg stage (Fig. II.4A and II.4B, Table II.1), with higher temperatures reducing the necessary time to complete the egg stage (t=-17.27, p<0.001 and t=-4.157, p<0.001, for 1- and 5-day exposed plants respectively). Eggs laid on plants previously exposed to ozone took, on average, longer to hatch (t=2.127, p=0.040 and t=2.017, p=0.062, for 1- and 5-day exposed plants respectively).

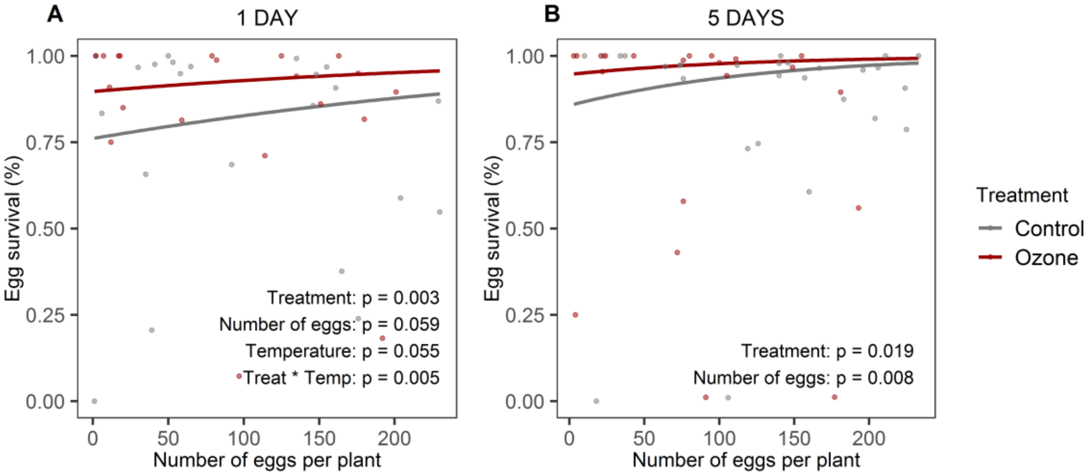


Figure II.3 – The effect of ozone and the number of eggs per plant on the survival rate of the eggs deposited on (A) 1-day exposed plants and (B) 5-days exposed plants. The lines represent the reported models' regression lines and the dots are the data points. The p-values for each predictor in the reported models are shown.

Temperature during the caterpillars development was the variable that affected caterpillar weight the most (t=22.950, p<0.001 and t=12.102, p<0.001, for caterpillars reared on 1- and 5-day exposed plants, respectively). Nine days into the caterpillar stage, no effect of ozone was observed on the weight of caterpillars reared on plants exposed for 1 day (Fig. II.4C). However, the caterpillars reared on plants exposed to ozone for 5 days were lighter than those reared on the corresponding control plants, but only when the temperature during the caterpillar stage was high (Fig. II.4D; Treatment x temperature during the caterpillar stage, t=-2.856, p=0.005).

Discussion

Exposure of plants to high levels of ozone has been shown to alter the interactions between plants and insect herbivores. However, the duration of the exposure and interactions with ambient temperature have not yet been considered as factors affecting plant-herbivore interactions. In this study, longer exposures to ozone affected the quality of *Sinapis arvensis* plants more strongly, with subsequent stronger effects on the interactions with the herbivore *Pieris brassicae*. *P. brassicae* butterflies avoided ozone exposed plants for oviposition. Despite a positive effect of ozone exposure on the survival of the eggs, the number of hatched caterpillars was lower on ozone-exposed plants and the caterpillars performed less well when feeding on them, particularly at higher ambient temperatures, a climate scenario that is likely to become more common in the future.

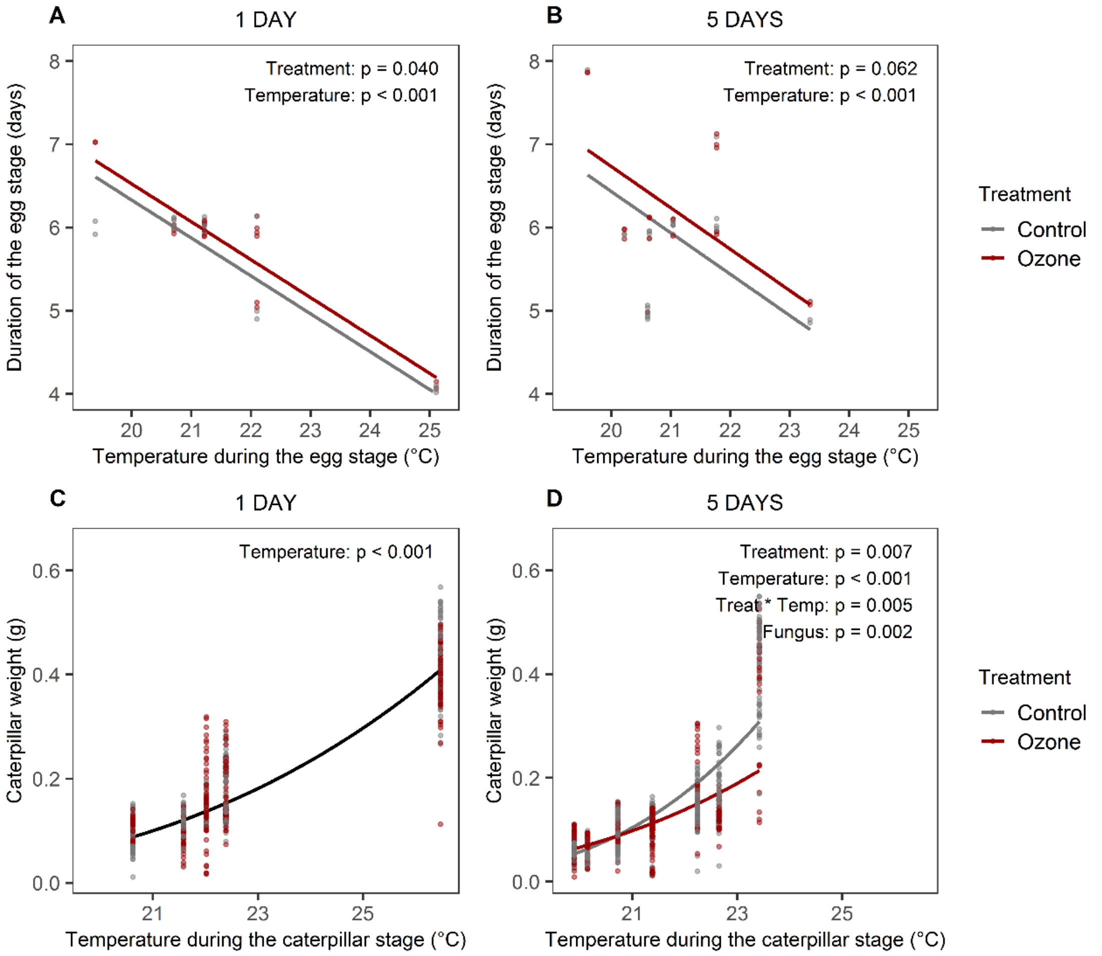


Figure II.4 – The effect of ozone and temperature on the duration of the egg stage (A and B) and the caterpillar weight (C and D). A and C refer to 1-day exposed plants and B and D correspond to the 5-days exposed plants. The lines represent the reported models' regression lines and the dots are the data points. In A and B data points were vertically jittered to improve visualization. The regression line in C is not colour-coded by treatment because ozone treatment was not a predictor in the reported model. The p-values for each predictor in the reported models are shown.

Increasing the duration of exposure of *S. arvensis* plants to ozone resulted in avoidance of these plants by *P. brassicae* butterflies when choosing oviposition sites. This is likely due to alterations in the chemical cues produced by the plant, particularly changes in the chemicals of the leaf boundary layer, that are often used in food plant acceptance (Hilker and Meiners, 2011). Our study is in accordance with some other studies addressing the plant-mediated effects of ozone on the oviposition preference of insect herbivores that show that the insects prefer to lay eggs on control plants (Cui et al., 2014; Jones and Coleman, 1988). In other studies, exposure of the plant to ozone had no effect on oviposition preference (Jackson et al., 1999; Peltonen et al., 2006), but, as we observed in the present study, this could be a matter of duration of exposure. In studies where the plants were presented to the insects during the exposure, some insects had a preference for laying eggs on control plants (Kopper and Lindroth, 2003) whilst others preferred ozone exposed plants (Jackson et al., 1999).

Plants may react to egg deposition by a hypersensitive response (Fatouros et al., 2016). In our study, the survival of the eggs was positively affected by ozone. We hypothesize that the exposure to an abiotic stress (ozone) prior to egg deposition may have inhibited a defence response from the plant in ozone-exposed plants, leading to a positive effect of ozone in the egg survival rate. Griese et al. (2017) showed that the expression or severity of the hypersensitive response does not increase with an increased number of eggs laid, but single-laid eggs are more susceptible to it than eggs laid in clusters and eggs laid in smaller clusters have a tendency to be more susceptible than eggs laid in bigger clusters. This is presumably because they are more vulnerable to desiccation. Although we did not specifically register survival per cluster, there was a positive correlation between the number of eggs per plant and the average number of eggs per cluster (r=0.75). We consider that an increased susceptibility of eggs in smaller clusters to a defence response may be the reason why, in this study, the egg survival rate was higher on plants with more eggs laid on them. Despite the positive effect of ozone on egg survival, the number of caterpillars per plant after hatching was still higher for control plants than for ozone-exposed plants, showing an overall negative effect of ozone.

The effect of ozone on caterpillar performance was also negative, but only for longer exposures and only when the caterpillars were reared at higher ambient temperatures. This gives rise to two non mutually exclusive hypothesis: 1) higher temperatures intensify the response of the plants to ozone with consequences to their nutritional value and/or to their level of toxicity, therefore affecting the caterpillars development or 2) higher temperatures increase the metabolic rate of the caterpillars, leading caterpillars that have similar weights at hatching to diverge faster, and therefore to show an indirect response to ozone. In any of these cases, the lower weight of caterpillars reared on plants exposed to ozone for 5 days may mean that the caterpillars are growing into lighter, weaker pupae or that the caterpillars will take longer to pupate, and therefore that their life cycle will be extended. The latter was the case in a study by Jondrup et al. (2002) , where they observed that caterpillars reared on ozone-sensitive plants exposed to ozone reached the same final weight, but took longer to pupate than the caterpillars reared on the control plants. On the other hand, Couture et al. (2012) observed that caterpillars showed decreased growth when fed foliage from trees growing under elevated ozone conditions. In Khaling et al. (2015) both phenomena occurred: caterpillars reared on ozone-exposed plants took longer to pupate and the pupae were lighter. If the reduced weight of the caterpillars reared on ozone-exposed plants shown here, translate into a longer caterpillar stage, together with the fact that the egg stage was also longer for eggs laid on ozone-exposed plants, the herbivores will have longer life cycles. Consequently, the number of generations produced per year may decrease and the predation or parasitism risk during the developmental stage may increase. Plant-mediated effects of ozone on caterpillar performance are not globally negative: Bolsinger et al. (1992) showed a higher relative growth rate of caterpillars when reared on plants exposed to ozone and Jackson et al. (2000) observed a tendency for increased growth of caterpillars fed with plants grown under elevated ozone conditions. Kopper et al. (2001) observed that ozone had no effect on the performance of caterpillars reared on trees growing under elevated ozone conditions and Jondrup et al. (2002) also saw no effect of ozone on caterpillars reared on resistant and wild type lines. When coupled with information about the nutritional state of the host, some studies suggest that alterations in caterpillar weight are related to changes in the nitrogen content of the host's leaves, whether the effect of ozone was negative (Couture and Lindroth, 2012; Couture et al., 2012; Khaling et al., 2015; Peltonen et al., 2010) or positive (Jackson et al., 2000). Interestingly, in no-choice tests, caterpillars either consumed similar amounts of plant material irrespective of plant treatment (Jondrup et al., 2002) or consumed more ozone-exposed plant material than control plant material (Abu ElEla et al., 2018; Jones and Coleman, 1988), which could indicate a mechanism to compensate for the reduction in nutritional value. However, in dual-choice feeding tests, herbivores also consumed more ozone-exposed plant material (Agathokleous et al., 2017; Khaling et al., 2015) suggesting that changes in palatability may be the reason for the modified consumption.

In this study, the butterflies laid more eggs on control plants than ozone-exposed plants, the same plants that later led to a better caterpillar performance. This is in agreement with the preference-performance hypothesis (Jaenike, 1978) which states that females choose oviposition sites that maximize the fitness of their offspring. By doing so, and having fairly mobile adults, *P. brassicae* may be able to escape the detrimental effects of ozone on its development as long as small scale variability in ozone damage exists. On the other hand, not being able to move, plants cannot escape ozone. They suffer stress from both ozone exposure and herbivory. We did not test for feeding preferences, but if Khaling et al.'s (2015) results on the increased consumption of ozone-exposed plant material would be applied in this situation, the fact that, as we observed, ozone-exposed plants had fewer caterpillars on them after hatching, may not be enough to compensate for the increased consumption. In our case ozone exposure seems to be a bad deal for both the plant and the herbivore. But even if one of them would be favoured by the exposure, the alterations in plant-herbivore interactions may affect the organisation of food webs, disturbing the balance of ecosystems.

Overall, the direction and strength of the herbivore response to ozone-exposed plants seem to vary between plant-insect systems. This variability may be caused by 1) different sensitivity to ozone between plant species, varieties or growth stages, 2) different susceptibility of the herbivores to the changes that ozone triggers in the plant or 3) different ozone exposure levels tested. The degree of sensitivity of a plant to ozone determines the exposure level that causes measurable changes in the plant which modify the plant's interaction with its herbivores. In this study, both the plant and the herbivore were sensitive enough so that ozone effects could be observed on the herbivore life cycle at the ozone levels studied. Our results also suggest that the effects of ozone on plant-insect interactions are cumulative, since ozone affected oviposition and caterpillar performance when the plants were exposed for 5 days but not when plants were exposed for 1 day. However, Agathokleous et al.(2019b) proposed that a plant does not respond linearly to ozone. A plant's response to ozone could also follow a hormetic model, with low doses being beneficial to plants and detrimental effects only being observed when the ozone dose exceeds the NOAEL (no-observed-adverse-effects level). In the present study, the detrimental effects on plant-insect interactions observed for an exposure of 120 ppb ozone, 6 h/day for 5 days reveals that this level of ozone is beyond the NOAEL for this plant-herbivore system.

Our results identify that relatively low concentrations of ozone affect plant-herbivore interactions. AOT40 (Accumulated Ozone exposure over a Threshold of 40 ppb) is an index defined by the European Union (EU) for the protection of the vegetation. It is determined by calculating the sum of the difference between hourly concentrations greater than 40 ppb and 40 ppb over a given period using hourly values measured between 8h00 and 20h00 CET. In the Directive on Ambient Air Quality (EU, 2008), the EU pointed to 6000 µg/m3.h (~3000 ppb.h) as the long-term objective to be reached. In our 5-day-long treatments, the calculated AOT40 is ~2300 ppb.h, a level well below EU's objective. However, as our results show, this level of exposure was already enough to cause damaging effects on the plants (visible injury) as well as to affect plant-herbivore interactions (oviposition and caterpillar performance). This points to the need of reviewing the European legislation on air quality, because currently it does not account for the damaging effect of acute ozone exposure, that seems to be important at least for annual plants like the one we used. Importantly, our data indicate that more frequent ozone peaks combined with higher temperatures, as predicted for a future with ongoing global warming and environmental pollution, will reinforce the negative effects of ozone on plant-herbivore interactions.

Conclusions

In summary, we showed that exposing *S. arvensis* to ozone affects several parameters of the life cycle of its herbivore *P. brassicae*. Our results reveal that a more severe exposure to ozone, especially when combined with higher temperatures, strengthens the effects of the pollutant on plant-herbivore interactions. Because plants vary in their sensitivity to ozone and herbivores vary in their susceptibility to changes in the plants, the alterations in plant-herbivore interations may vary in strength and direction between plant-herbivore systems, affecting the organisation of food webs and possibly disturbing the balance of ecosystems. This accentuates the need to implement measures to reduce the emission of precusors that could lead to ozone peaks such as the ones tested here, particularly in parts of the world where the use of fossil fuels is still increasing.

Supplementary material to chapter II

Table SII.1 – Ozone concentration (mean ± standard deviation) and accumulated exposure to ozone (AOT40) in the ozone chamber between 11h00 and 17h00, during the treatment days of each round.

Round - Duration of treatment	[O3] (ppb)	AOT40 (ppb.h)
1 - 1 day	112 ± 14	437
2 - 1 day	113 ± 11	437
3 - 1 day	119 ± 9	472
4 - 1 day	119 ± 10	473
5 - 1 day	122 ± 10	494
6 - 1 day	120 ± 11	479
1 - 5 days	117 ± 8	2298
2 - 5 days	116 ± 9	2274
3 - 5 days	116 ± 9	2267
4 - 5 days	114 ± 10	2209
5 - 5 days	114 ± 9	2230
6 - 5 days	114 ± 9	2231
7 - 5 days	117 ± 9	2312

Table SII.2 – Summary of the models with ΔAICc < 2 for the number of eggs laid per plant in dual-choice assays

				Predictors							
Oviposition preference	Model	ΔAICc	weight	Treatment		Fungal Infection		Plant height		Treat * Fungal Inf	
				z	P	z	P	z	P	z	P
1 day	Model 1	0.00	0.301	----------	----------	-2.346	0.019	----------	----------	----------	----------
	Model 2	0.17	0.276	----------	----------	-2.713	0.007	1.447	0.148	----------	----------
5 days	Model 1	0.00	0.241	-1.830	0.067	-2.088	0.037	----------	----------	----------	----------
	Model 2	1.05	0.142	----------	----------	-1.880	0.060	----------	----------	----------	----------
	Model 3	1.15	0.135	-1.665	0.100	----------	----------	----------	----------	----------	----------
	Model 4	1.65	0.105	----------	----------	----------	----------	----------	----------	----------	----------

Table SII.3 – Summary of the models with ΔAICc < 2 for the number of caterpillars on the plants

				Predictors					
Caterpillars on plants	Model	ΔAICc	weight	Treatment		Fungal Infection		Treat * Fungal Inf	
				z	P	z	P	z	P
1 day	Model 1	0.00	0.515	----------	----------	-2.248	0.025	----------	----------
	Model 2	1.86	0.203	----------	----------	----------	----------	----------	----------
5 days	Model 1	0.00	0.499	-2.209	0.027	----------	----------	----------	----------
	Model 2	1.68	0.215	-2.242	0.025	-0.832	0.405	----------	----------

Table SII.4 – Summary of the models with ΔAICc < 2 for the duration of the egg stage

				Predictors									
Duration of the egg stage	Model	ΔAICc	weight	Treatment		Temperature		Number of eggs		Treat * Temp		Treat * Nr of eggs	
				t	P	t	P	t	P	t	P	t	P
1 day	Model 1	0.00	0.361	2.127	0.040	-17.269	< 0.001	-------	-------	-------	-------	-------	-------
	Model 2	0.81	0.240	1.496	0.143	-12.650	< 0.001	-------	-------	-1.333	0.190	-------	-------
5 days	Model 1	0.00	0.385	2.107	0.062	-4.157	< 0.001	-------	-------	-------	-------	--------	--------
	Model 2	1.80	0.157	1.781	0.112	-4.151	< 0.001	-0.817	0.430	--------	-------	--------	--------

Table SII.5 – Summary of the models with ΔAICc < 2 for the egg survival rate

				Predictors									
Egg survival rate	Model	ΔAICc	weight	Treatment		Temperature		Number of eggs		Treat * Temp		Treat * Nr of eggs	
				z	P	z	P	z	P	z	P	z	P
1 day	Model 1	0.00	0.275	2.962	0.003	1.923	0.055	1.885	0.059	-2.796	0.005	--------	--------
	Model 2	0.24	0.244	-1.636	0.102	--------	--------	-1.057	0.291	--------	--------	2.778	0.005
	Model 3	0.4	0.226	3.876	< 0.001	1.958	0.050	-3.609	< 0.001	--------	--------	--------	--------
	Model 4	1.7	0.117	-1.592	0.111	1.135	0.256	-0.966	0.334	--------	--------	2.724	0.006
	Model 5	1.92	0.105	1.363	0.173	1.655	0.098	-0.070	0.944	-1.557	0.119	0.984	0.325
5 days	Model 1	0	0.359	2.352	0.019	--------	--------	2.633	0.008	--------	--------	--------	--------
	Model 2	1.42	0.176	2.316	0.021	-1.037	0.300	2.633	0.008	--------	--------	--------	--------
	Model 3	1.69	0.154	1.611	0.107	--------	--------	1.892	0.059	--------	--------	-0.886	0.375

Table SII.6 – Summary of the models with ΔAICc < 2 for the caterpillar weight

				Predictors													
Larval weight	Model	ΔAICc	weight	Treatment		Temperature		Number of eggs		Fungal infection		Treat * Temp		Treat * Nr of eggs		Treat * Fung Inf	
				t	P	t	P	t	P	t	P	t	P	t	P	t	P
1 day	Model 1	0.00	0.267	-----	-----	22.950	< 0.001	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
	Model 2	1.49	0.127	-----	-----	7.327	< 0.001	-----	-----	-0.720	0.474	-----	-----	-----	-----	-----	-----
	Model 3	1.73	0.112	-0.532	0.596	22.829	< 0.001	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----
	Model 4	1.95	0.101	-----	-----	22.194	< 0.001	-0.277	0.783	-----	-----	-----	-----	-----	-----	-----	-----
5 days	Model 1	0.00	0.418	2.760	0.007	12.102	< 0.001	-----	-----	-3.141	0.002	-2.856	0.005	-----	-----	-----	-----
	Model 2	1.50	0.198	2.839	0.005	11.934	< 0.001	-----	-----	-3.005	0.003	-2.926	0.004	-----	-----	0.714	0.477

Table SII.7 – Number of replicates for each research question.

Duration of the treatment	1 day		5 days	
Treatment	Ct	O_3	Ct	O_3
Plant injury	48	48	56	56
Number of laid eggs	35	35	45	45
Duration of the egg stage	22	20	27	21
Egg survival rate	23	20	28	21
Number of caterpillars per plant	35	35	45	45
Caterpillar weight	330	374	531	468

Ct-control treatment

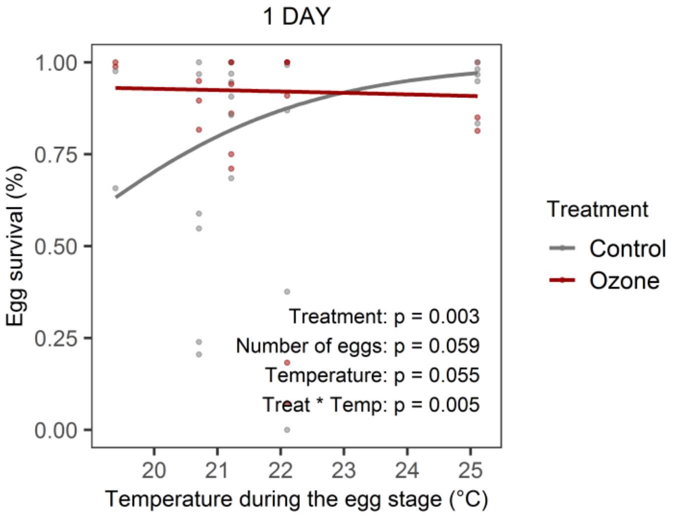
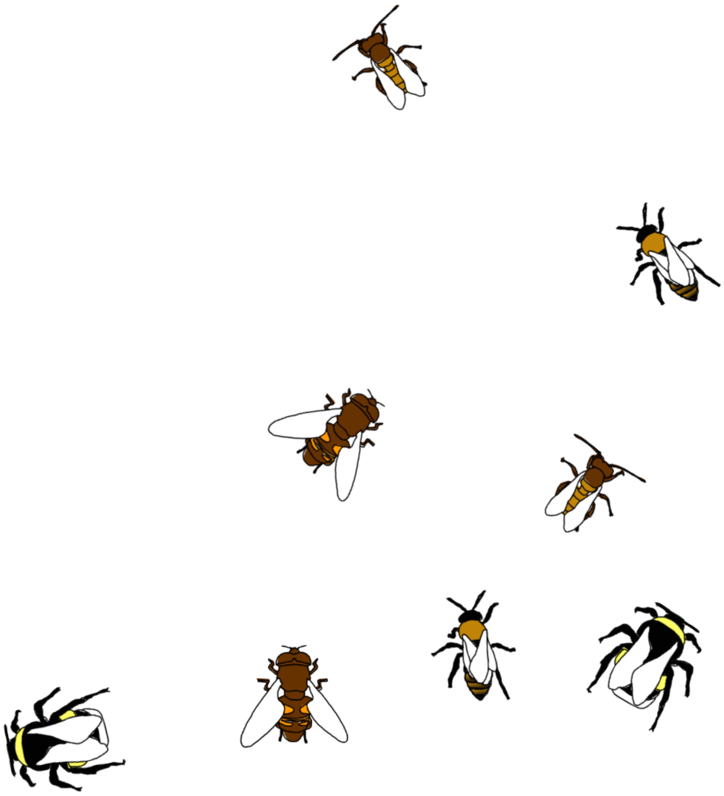


Figure SII.1 – The effect of ozone and temperature on the survival rate of eggs laid on 1-day treated plants. The lines represent the reported model's regression lines and the dots are the data points. The p-values for each predictor in the reported model are shown.



Chapter III

Effects of ozone stress on flowering phenology, plant-pollinator interactions and plant reproductive success

Tropospheric ozone is a highly oxidative pollutant with the potential to alter plant metabolism. The direct effects of ozone on plant phenotype may alter interactions with other organisms, such as pollinators, and, consequently, affect plant reproductive success. In a set of greenhouse experiments, we tested whether exposure of plants to a high level of ozone affected their phenological development, their attractiveness to four different pollinators (mason bees, honeybees, hoverflies and bumblebees) and, ultimately, their reproductive success. Exposure of plants to ozone accelerated flowering, particularly on plants that were growing in autumn, when light and temperature cues, that commonly promote flowering, were weaker. Simultaneously, there was a tendency for ozone-exposed plants to disinvest in vegetative growth. Plant exposure to ozone did not substantially affect pollinator preference, but bumblebees had a tendency to visit more flowers on ozone-exposed plants, an effect that was driven by the fact that these plants tended to have more open flowers, meaning a stronger attraction signal. Honeybees spent more time per flower on ozone-exposed plants than on control plants. Acceleration of flower production and the behavioural responses of pollinators to ozone-exposed plants resulted in retained reproductive fitness of plants pollinated by bumblebees, honeybees and mason bees, despite the negative effects of ozone on plant growth. Plants that were pollinated by hoverflies had a reduction in reproductive fitness in response to ozone. In a natural setting, acceleration of flowering by ozone might foster desynchronization between plant and pollinator activities. This can have a strong impact on plants with short flowering periods and on plants that, unlike wild mustard, lack compensatory mechanisms to cope with the absence of pollinator activity in the beginning of flowering.

This chapter is published as **Duque, L.**, Poelman, E.H., Steffan-Dewenter, I., **2021**. *Effects of ozone stress on flowering phenology, plant-pollinator interactions and plant reproductive success*. Environmental Pollution 272, 115953..

Introduction

Animal pollination, in which animals transfer pollen grains from the male to the female reproductive organs of the plants, is of major importance for plant reproductive success: animals are estimated to pollinate 87.5% of the world's angiosperms (Ollerton et al., 2011) and 75% of the main food crops rely at least to some extent on animal pollination (Klein et al., 2007). Most animal pollination services are provided by insects. With a diet that depends almost exclusively on floral resources, such as nectar and pollen, bees are at the top of the pollinators' list, which also includes hoverflies, butterflies, moths, flies, beetles and vertebrates (Kearns et al., 1998). Plant-pollinator interactions are considered to be at risk due to multiple drivers of global change, such as climate change and land-use intensification (Vanbergen et al., 2013). Global change is modifying plant-pollinator interactions by reducing the abundance and diversity of pollinators (Byers, 2017; IPBES, 2016; Powney et al., 2019), by introducing phenological mismatches between the players (Burkle et al., 2013; Byers, 2017; Memmott et al., 2007; Visser and Gienapp, 2019) or by decreasing the attractiveness of the plants to the pollinators (Burkle and Runyon, 2017; Dotterl et al., 2016; Farre-Armengol et al., 2015; Girling et al., 2013; Lusebrink et al., 2015; Riffell et al., 2014). Air pollution in general, and ozone in particular, have received little attention in the context of plant-pollinator interactions, but should not be disregarded as a threat.

In spite of the efforts to reduce pollutant emissions in some parts of the world (EPA, 1990; EU, 2008; The Chinese State Council, 2013) air pollution is still an important component of global change. Out of all air pollutants, ozone has the strongest negative impacts on vegetation, due to its high oxidation potential. It is a secondary pollutant, formed by the reaction between nitrogen oxides and volatile organic compounds (VOCs) in the presence of sunlight. Therefore, tropospheric ozone concentrations are highly dependent both on precursor emissions and climate/weather conditions. Control of precursor emissions has contributed to a considerable decrease in tropospheric ozone concentrations in Europe and North America (Lin et al., 2017; Yan et al., 2019), while in China ozone concentrations are still witnessing a rapid increase (Lu et al., 2020). In fact, modelling approaches predict an increase in exposure to ozone (Fuhrer et al., 2016) and an increase in the number of high ozone episodes (Lei et al., 2012) in many parts of the world in the coming years. Controlling emissions is not being as successful in reducing tropospheric ozone concentrations as desired, due both to long-range transport of its precursors (Lin et al., 2017) and to the changing climate (Lin et al., 2020). Nitrogen oxides are often transported from urban to rural areas. Here, the VOCs needed for ozone production are supplied by the vegetation and are not a limiting factor, making ozone concentrations typically higher in rural areas (Fitzky et al., 2019; Yan et al., 2018). On the other hand, vegetation also acts as a sink for ozone, removing it from the troposphere through stomatal uptake or dry deposition on leaf surfaces (Fitzky et al., 2019).

The process of ozone removal by vegetation is, however, detrimental to plants. The most commonly reported effect of tropospheric ozone on plants is the presence of visible injury symptoms, mainly chlorotic/necrotic spots on the leaves, but other effects may be of higher importance, such as the reduction of biomass (Mills et al., 2011). A recent study on the impacts of tropospheric ozone on crops estimates that at the ozone levels observed in 2010-2012 the global yield losses due to ozone added up to 7.1 % for wheat, 12.4 % for soybean, 4.4 % for rice and 6.1 % for maize (Mills et al., 2018). Assuming that the current legislation scenario will be fully implemented by 2030, further losses are to be expected (Van Dingenen et al., 2009). Besides the direct effects on plants, ozone has the potential to affect interactions of plants with insects. Studies on plant-herbivore interactions have shown that herbivores avoid laying eggs and caterpillars take longer to hatch on ozone-exposed plants (Duque et al., 2019), and that caterpillars grow slower or lighter on ozone-exposed plants, due to their lower nutritional value (Couture et al., 2012; Duque et al., 2019; Jondrup et al., 2002; Khaling et al., 2015; Peltonen et al., 2010).

Although there is now ample evidence that ozone can affect plant interactions with antagonists, such as insect herbivores, we still largely lack understanding on how ozone affects interactions of plants with mutualists, such as pollinators. Exposure to ozone changes the bouquet of flower scents that pollinators use while searching for food sources, which can affect the attractiveness of plants to pollinators. Ozone can either modulate the mixture of floral volatiles emitted by the plants (Saunier and Blande, 2019) or react with these chemical compounds so that the floral blend is altered in the atmosphere (Farre-Armengol et al., 2016). Plant-pollinator interactions may also be affected because ozone has the potential to change the quality and quantity of floral rewards (pollen and nectar) (Stabler, 2016). Exposure to ozone can also alter flowering phenology, with studies reporting accelerated (Drogoudi and Ashmore, 2000; Hayes et al., 2012) or delayed flowering (Amundson et al., 1986; Rämö et al., 2007), and reduced number of flowers (Hayes et al., 2012; Rämö et al., 2007), but responses of flower visitors and effects on plant reproduction are mainly unknown. Pollinators show preferences for a given floral bouquet to the detriment of others and this behaviour is pollinator species-specific (Larue et al., 2016). Similarly, when it comes to nectar quality, pollinators also differ in their preferences and requirements (Petanidou, 2005). Therefore, it is reasonable to assume that the response of pollinators to ozone-exposed plants may also differ among pollinators.

In this study, we aim to assess whether exposure of wild mustard (*Sinapis arvensis)* plants to ozone affects plant phenology, the interactions between the plants and their pollinators, and ultimately the plants' reproductive success. Wild mustard, is an important weed of field crops, common to most temperate regions of the world (Warwick et al., 2000). It is an annual plant with indeterminate growth habit, continuing to produce flowers when the reproductive success is low (Warwick et al., 2000). It is self-incompatible, depending on a wide range of insects for pollination (Mulligan and Bailey, 1975). It is a pollinator generalist, visited mostly by bees (both social and solitary), hoverflies, but also by some butterflies, beetles and other flies (Fogg, 1950). In a set of 4 greenhouse experiments, we exposed wild mustard plants to 120 ppb ozone for 6 hours/day over a period of 5 days and, subsequently, measured responses of four different pollinator species. We specifically addressed the following research questions: 1) Does ozone affect the flowering and vegetative patterns of the plant? 2) Does a prior exposure of plants to ozone affect the foraging behaviour of the pollinators, and does proximity in time to the exposure alter this effect? 3) Is the reproduction of wild mustard plants affected by exposure to ozone?

Material and methods

In four separate experiments performed between April and December 2019, we tested the response of one of four pollinator species to plants previously exposed to a high level of ozone. In each of the experiments we assessed how plant phenological development, pollinator behaviour and plant reproductive success are affected by ozone (Figure III.1; Table SIII.1).

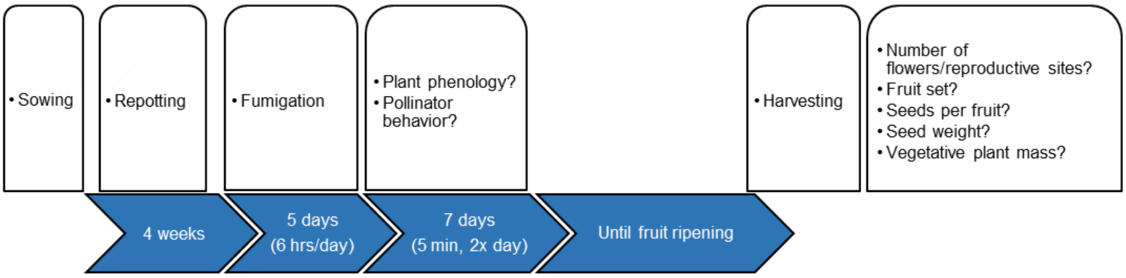


Figure III.1 – Scheme of the experiments

Plant material

Wild type *Sinapis arvensis* seeds were provided by the Botanical Garden of Konstanz, Germany. The plants were grown in an insect-proof greenhouse chamber in 18x18 cm pots with a 2:1 mixture of peat-based substrate (Einheits Erde CL ED 73) and sand (Hamann Filtersand 0,7-1,25 mm). High-pressure sodium lamps were on, between 5:00 and 21:00 CET, whenever the light intensity outside the greenhouse dropped below 20 kLux. The greenhouse conditions were set to: 1) temperature - 23°C/18°C and 2) relative humidity - 50%/80% during light/dark periods respectively. However, the greenhouse does not possess an effective cooling system, meaning that in warm, sunny days the temperature can raise above the set values. During these experiments, a maximum of 35.5 °C was reached during the day.

In a preliminary greenhouse experiment, we subjected flowering plants of the same population used for this study to three different treatments: hand cross-pollination, hand self-pollination and no pollination. The percentage of flowers setting fruit in each treatment (52%, 6% and 2% respectively) revealed that the reproduction of this wild mustard population is highly dependent on cross-pollination.

Plant fumigation

Fumigation was performed in a customized ozone-exposure system with two fumigation chambers (Duque et al., 2019). Fumigation started when the plants were four weeks old and had started flower bud development. Because of delayed development, the plants in the last round were fumigated when they were 4 weeks and 3 days old. The fumigation treatment consisted of exposing the plants to either 1) 0 ppb of ozone (clean air or control) or 2) 120 ppb of ozone, for 6 hours/day (11:00-17:00 CET) and to clean air the rest of the day (Table SIII.1). Fumigation lasted for 5 days. This level of exposure is known to cause damage on plants and affect plant-herbivore interactions (Duque et al., 2019). 120 ppb is considered a hazardous tropospheric ozone concentration that is sometimes exceeded during high ozone episodes (Lei et al., 2012). For each fumigation round, eight plants were introduced in each chamber. Two fumigation rounds were performed for each pollinator species, in a total of 16 plants x treatment x pollinator.

Pollinators

Four separate experiments were performed with four different pollinators to assess if plant exposure to ozone affected the pollinators' foraging behaviour. Pollinator species were chosen on the basis of covering a spectrum of different life-styles/behaviours and for their accessibility. We used a solitary bee, the mason bee *Osmia bicornis*; two social bees: the honeybee *Apis mellifera* and the bumblebee *Bombus terrestris*; and a hoverfly, *Eristalis tenax*.

The Department of Animal Ecology and Tropical Biology of the University of Würzburg maintains an outdoors area with trap-nesting solitary bee populations and honey bee colonies. In the beginning of April 2019 *O. bicornis* cocoons were retrieved from nests and stored at 4°C until they were moved to ambient temperature for emergence. The mason bees were gradually released in the greenhouse chamber, with 118 bees in total. One small colony of *A. mellifera* with two brood combs was placed in the greenhouse. We used a mini colony of *B. terrestris* (Biobest, Belgium) with ~30 workers. Since the bumblebees were visibly destroying some flowers likely due to food stress, in the first days, they were only allowed to pollinate during the periods when we monitored the flower visitation. *E. tenax* pupae (Polyfly S.L., Spain) emerged in an insect-rearing tent and were fed sugar, water and a pollen mixture, before being gradually released in the greenhouse, with 128 hoverflies in total.

Flower visitation

Following each fumigation round, the plants were moved to a separate greenhouse chamber and plants from each treatment were placed alternately, with 80 cm between plants.

Flower visitation was observed separately for each plant during the 7 days following fumigation, in 2 periods of 5 minutes per day (one in the morning and a second one in the beginning of the afternoon). After this 7-day period, a second round of fumigated plants was introduced in the same greenhouse chamber for flower visitation observations. The pollinators remained in the greenhouse chambers beyond the periods of observations, until circa 90% of the plants had finished flowering, so that virtually all flowers had the opportunity of being pollinated.

During the flower visitation observations we recorded the number of visitors, the number of flowers visited per pollinator and, when possible, the duration of visits. Pollinators, particularly hoverflies, often sat inactive on the plants. Only the periods of time when pollinators were active on the flowers were included in the duration of the visits. 923 visits were observed during the monitoring period. We recorded the duration of 548 of those visits. In the four cases when the number of flowers visited by a pollinator was not possible to count, this number was estimated based on the other visits performed in the same time period.

Plant phenology and reproduction

During the flower-visitation observation period, the number of open flowers in each plant was counted daily. After ripening of pods, the plants were left to dry in a dry greenhouse compartment. The shoot of the dry plants was cut and weighed. All reproductive sites were counted, distinguishing those that did not set fruit from those that did and those that were produced in the terminal inflorescence from those produced by the rest of the plant. With these data we calculated reproductive success rate = number of reproductive sites that set fruit / (number of reproductive sites that set fruit + number of reproductive sites that did not set fruit). We randomly sampled 20 pods (whenever possible) from each plant (10 from the terminal inflorescence and 10 from the rest of the plant). The seeds produced per pod were counted and 100 seeds (whenever possible) were weighed. Total number of seeds produced per plant and total seed weight were estimated as follows: total number of seeds per plant = number of pods produced per plant x average number of seeds per pod; total seed weight = estimated total number of seeds per plant x average seed weight. To get the vegetative dry mass of each plant, the estimated total seed weight was subtracted to the dry weight of the plant.

Statistical analysis

All statistical analyses were performed using R (version 4.0.1)(R Core Team, 2020). To test all our research questions we fitted (generalized) linear mixed effects models using the glmmTMB function in the glmmTMB package (version 1.0.1) (Brooks et al., 2017), that can deal with zero-inflated data whenever necessary (see supplementary materials, Fitted models and Results tables, for details on the models tested and their results). We used the Dharma package (version 0.3.1)(Hartig, 2020) to evaluate the fit of the models. Type 2 anova test results were obtained for all our models using the car package (version 3.0.8) (Fox and Weisberg, 2019). We used the package emmeans (version 1.4.7)(Lenth, 2020b) to perform a post-hoc test to disentangle a significant interaction between treatment and experiment on the plants' flowering patterns (supplementary materials table S3).

When testing whether ozone affects the flowering patterns and the vegetative development of the plants, we included the experiment as a possible explanatory variable because we suspected that the conditions observed during the different experiments, particularly in terms of the amount of photosynthetically active radiation, could have an impact on flowering phenology. Since this was the case, subsequent analyses were performed separately for each experiment. Plants that did not start to flower before the end of the flower visitation observation period were only included for the purposes of testing the onset of flowering and were excluded from further analysis.

Results

The onset of flowering was accelerated by ozone ($\chi^2 = 13.1$, df = 1, $p < 0.001$, Fig. III.2A, Table SIII.2) and differed between experiments ($\chi^2 = 98.0$, df = 3, $p <$

0.001, Fig. III.2A). A treatment x experiment interaction ($\chi^2 = 19.4$, df = 3, $p < 0.001$, Fig. III.2A) reveals that acceleration of the onset of flowering by ozone only occurred in the two autumn experiments (t-ratio= 5.478, $p < 0.001$ and t-ration = 1.976, $p = 0.049$, for experiments 3 and 4 respectively, Table SIII.3). Therefore, in those experiments, the number of flowering plants was over-represented by ozone-exposed plants during the flower visitation observation period. Amongst the plants that were flowering, the number of open flowers increased during the period of observations ($\chi^2 = 307.5$, df = 1, $p < 0.001$, Fig. III.2B) and differed between experiments ($\chi^2 = 43.7$, df = 3, $p < 0.001$, Fig. SIII.1), but was not affected by ozone ($\chi^2 = 1.9$, df = 1, $p = 0.165$, Fig. III.2B). Overall, each plant produced 1246 ± 535 flowers and this number was not affected by ozone ($\chi^2 = 2.6$, df = 1, $p = 0.110$, Fig. III.3A, Table SIII.4). However, exposure to ozone resulted in a modest decrease in the above-ground vegetative plant mass ($\chi^2 = 3.0$, df = 1, $p = 0.084$, Fig. III.3B, Table SIII.4). The plants' investment in vegetative mass and in reproductive structures (flowers) was different between experiments ($\chi^2 = 166.2$, df = 3, $p < 0.001$ and $\chi^2 = 121.2$, df = 3, $p < 0.001$, respectively, Fig. III.3).

The number of flower visits increased with time as a response to the increasing number of open flowers (Fig. III.4, Table SIII.5). Bumblebees tended to perform more flower visits during the 10-minute observation periods on ozone-exposed plants than on control plants ($\chi^2 = 2.9$, df = 1, $p = 0.088$). However, when correcting for the absolute number of open flowers and its proportional abundance within each time period, there were no differences in pollinator visitation as measured by the number of flower visits per plant (Table SIII.6, Fig. SIII.2) or the number of visitors received per plant (Table SIII.7), revealing that the positive effect of ozone on bumblebee flower visitation was in fact an indirect effect of ozone exposure. Ozone had no effect on the number of visits performed by the other pollinators (Tables SIII.5-SIII.8).

Hoverflies spent 10.8 ± 7.5 s per flower, while honeybees spent 3.4 ± 2.3s, the mason bees spent 3.3 ± 2.0 s and the bumblebees 2.6 ± 2.0 s. Honeybees spent more time per flower on ozone-exposed plants than on control plants ($\chi^2 = 7.7$, df = 1, $p = 0.010$, Fig. III.5), while for the other pollinators there was no effect of the ozone treatment on the time spent per flower (Table SIII.9).

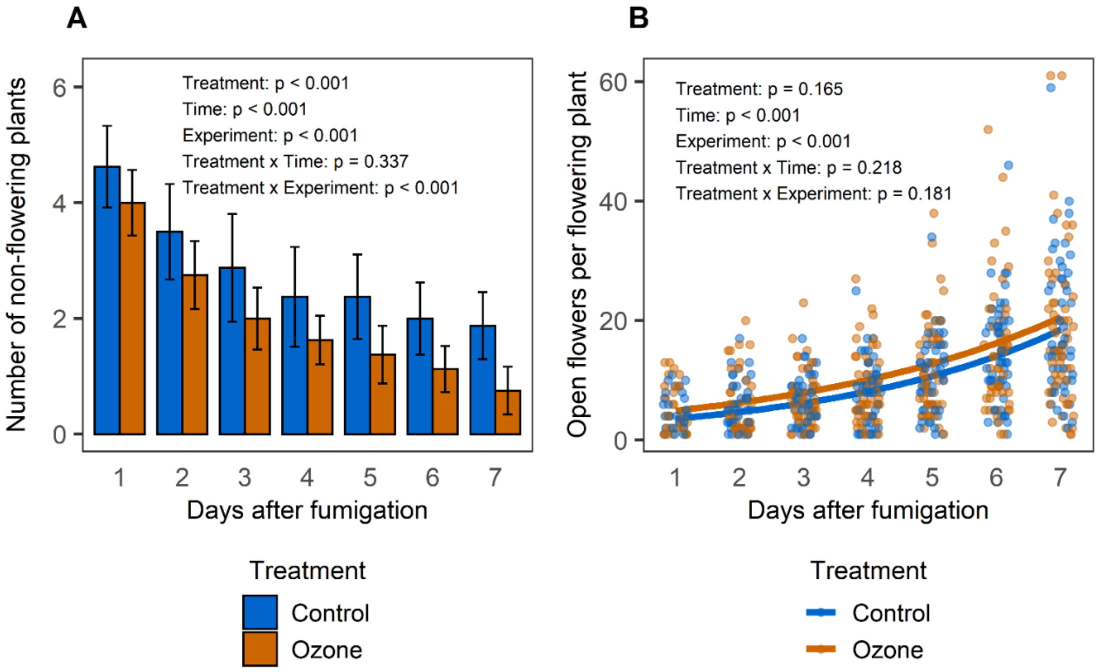


Figure III.2 – The flowering patterns of plants, in the 7 days after fumigation, as affected by ozone. A) Ozone exposure accelerated the flowering onset but B) the number of flowers in the flowering plants was not affected. P-values are presented for all the fixed variables included in the 2 components of the model tested: A) the zero-inflation model and B) the conditional model. A) shows the means and standard errors and B) shows the regression lines of the conditional model tested and the data points. Data points were jittered to improve visualization.

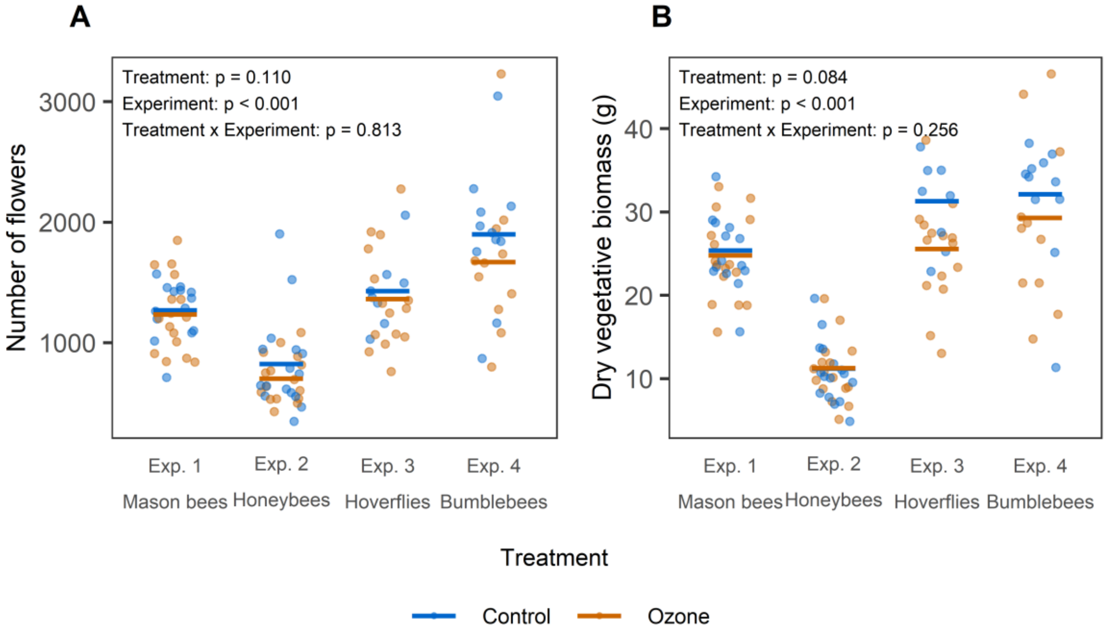


Figure III.3 – The effect of ozone on the plants' investment in A) reproductive and B) vegetative structures. The dots are the data points and the dashes represent the means as provided by the models. P-values are presented for all fixed variables included in the models.

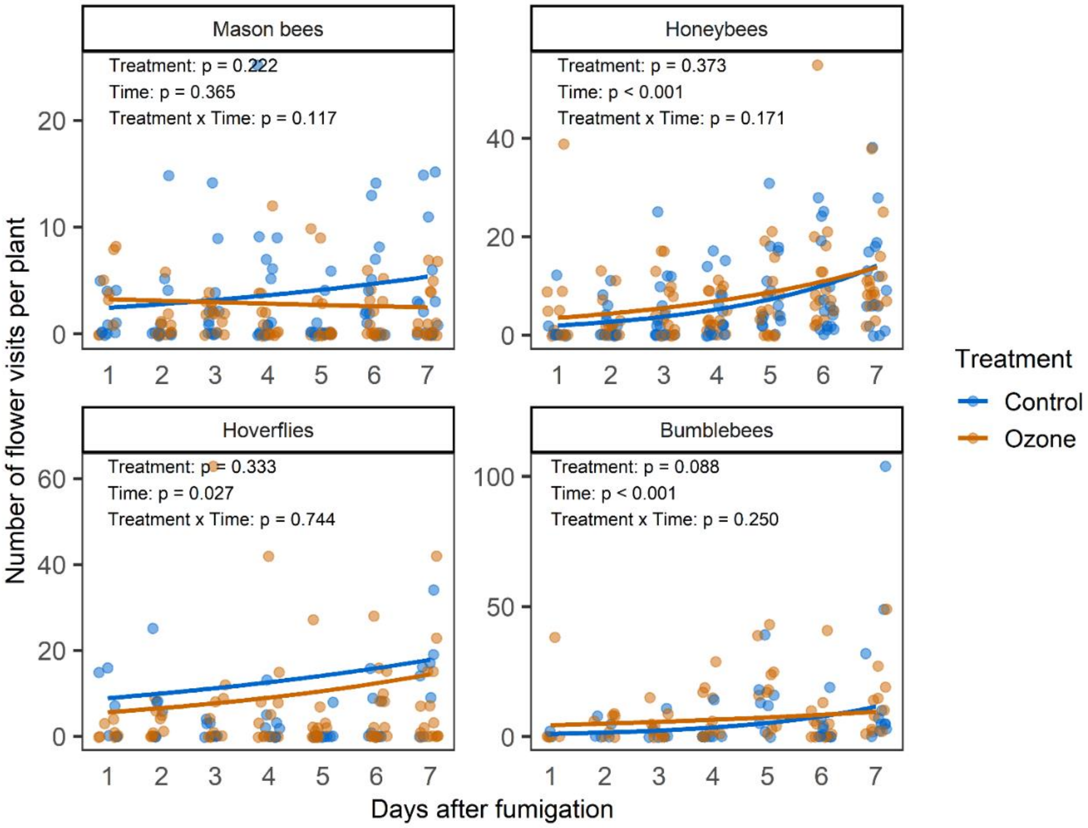


Figure III.4 – The effect of ozone on the number of flower visits a plant receives in 10-min periods on the 7 days following fumigation for four different pollinators. Models were not corrected for the amount of open flowers available for pollination (see Fig. S2 for the corrected models). The lines represent the regression lines of the models tested and the dots are the data points. Data points were jittered to improve visualization. P-values are presented for all fixed factors included in the model.

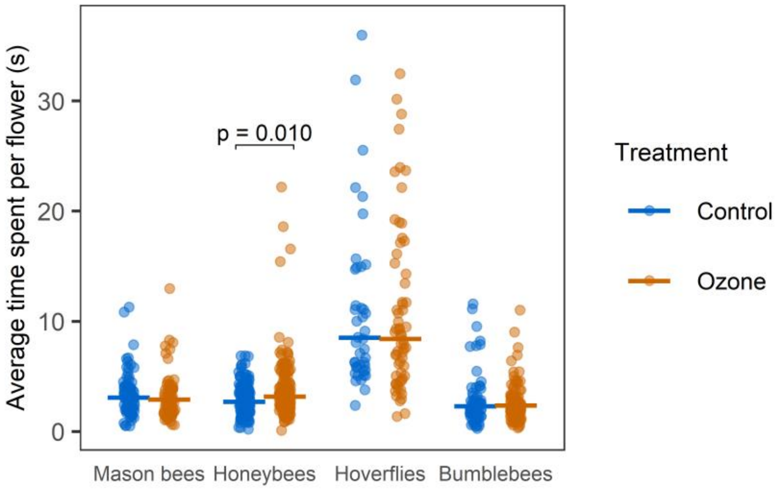


Figure III.5 – The effect of ozone on the time spent per flower by four different pollinators. The dots are the data points and the dashes represent the means as provided by the models. The p-value for the effect of ozone is presented for the honeybees, the only pollinator that altered the time spent per flower in function of the ozone treatment.

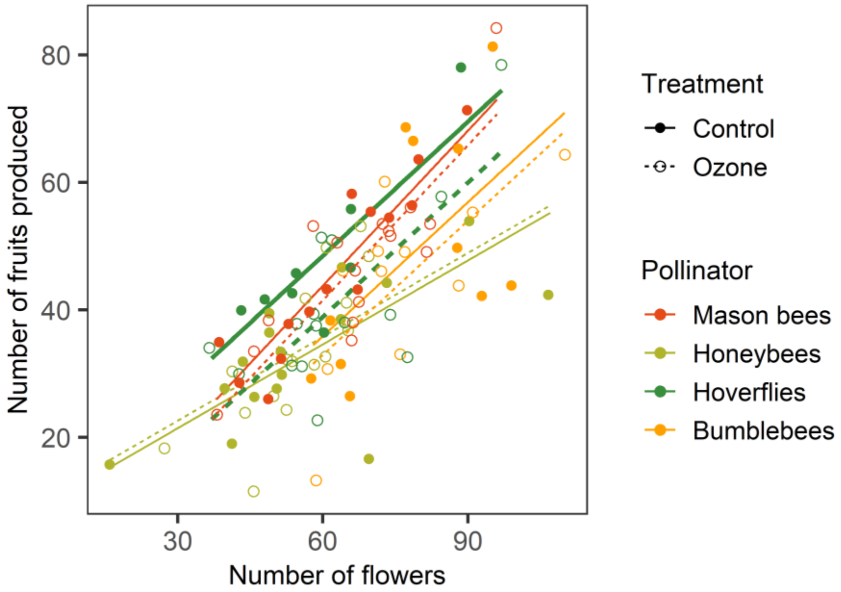


Fig. 6- The effect of ozone on the fruit set of the terminal inflorescence of plants pollinated by four different pollinators. The dots are the data points and the lines represent the regression lines of the models tested. The effect of ozone on fruit set was only significant for the plants pollinated by the hoverflies (p = 0.009).

Ozone exposure reduced the number of flowers developing fruits and the reproductive success rate of the hoverfly-pollinated plants (χ^2 = 4.8, df = 1, p =0.029 and χ^2 = 2.8, df = 1, p =0.092, respectively, Tables SIII.10 and SIII.11). The effects of ozone were stronger when considering only the terminal inflorescence (χ^2 = 6.9, df = 1, p =0.009 and χ^2 = 5.2, df = 1, p =0.022, for fruit set and success rate respectively, Fig. III.6). The effect of ozone on these parameters of plant reproduction was not significant for plants pollinated by the other 3 species. Each of the fruits produced by the plants had between 1 and 20 seeds (median 11) and this was not affected by ozone (Table SIII.12). The average seed weight of honeybee-pollinated plants was lower in plants exposed to ozone than in control plants both for the terminal inflorescence (χ^2 = 3.2, df = 1, p = 0.075) and for the rest of the plant (χ^2 = 8.7, df = 1, p = 0.003). Ozone had no effect on the individual seed weight of plants pollinated by the other species (Table SIII.11). Plant fitness as measured by the estimated total number of seeds produced was only affected by ozone for plants pollinated by hoverflies (χ^2 = 5.3 df = 1, p = 0.021, Table SIII.11).

Discussion

Exposure of wild mustard plants to a high ozone level during the flower formation stage resulted in an acceleration of flowering, increasing the representation of open flowers on ozone-exposed plants in the beginning of the flowering period. Moreover, ozone did not substantially affect flower visitation, but bumblebees visited more flowers on ozone-exposed plants, which was likely driven by the fact that these plants had more flowers available for pollination. Honeybees spent more time per flower on plants exposed to ozone. Acceleration of flowering and the behavioural responses of pollinators to ozone-exposed plants resulted in retained reproductive success of plants pollinated by bumblebees, honeybees and mason bees. On the other hand, hoverfly-pollinated plants exposed to ozone showed reduced fruit and seed set, resulting in reduced reproductive success under ozone exposure.

Although exposure to ozone accelerated the onset of flowering, it did not affect the overall number of flowers produced by the plants. Induction or acceleration of flowering in response to stress has been described before (Pashalidou et al., 2013; Takeno, 2016), but there is a very limited amount of information on the effects of ozone stress on flowering patterns. Hayes et al. (2012) observed that the time to reach peak flowering of *Lotus corniculatus* was reduced by 6 days as the exposure to ozone increased from a concentration of 30 to 70 ppb, but the number of open flowers during peak flowering was not affected. In the same study, they found an overall lower number of flowers with increasing ozone concentration in *Campanula rotundifolia* and *Scabiosa columbaria* plants. Rämö et al. (2007) also observed a reduction in the number of *C. rotundifolia* flowers produced, in response to ozone, but in this case coupled with a delay in flowering. Changing the flowering patterns may have consequences for both the plant and its pollinators. Shifting the onset of flowering may lead to desynchronization between plant and pollinator activity, reducing both plant and pollinator fitness (Schenk et al., 2018) Climate change is considered a threat for pollination because it may result in mismatches between plants and pollinators (Byers, 2017). Some studies have reported that plants tend to accelerate their phenology more in response to warming than pollinators (Forrest and Thomson, 2011; Kudo and Ida, 2013) and ozone may add to the mismatch by further advancing flowering and leaving the plants without interaction partners for longer periods, reducing their fitness. The general direction, however, seems to be of a faster acceleration of insect phenology than plant phenology in response to warming (Thackeray et al., 2016), in which case, ozone may counteract temperature effects and re-establish synchronization. In addition, pollinators themselves may be able to induce the onset of flowering when faced with low availability of pollen resources and increase synchronisation of flowering time with pollinator presence (Pashalidou et al., 2020). Plant-pollinator temporal mismatches are of particular concern for highly specialized plant-pollinator interactions, for plants and pollinators that have a reduced activity period and for plants and pollinators that are active in the extremes of the pollinating season (Burkle et al., 2013; Forrest and Thomson, 2011). Being a pollinator generalist that blooms in the middle of the pollinating season and has a flowering period that lasts for a few weeks, wild mustard is unlikely to be at risk of desynchronization with its pollinators. Other costs of acceleration of flowering, which may have consequences for reproduction, are reduced allocation of resources to vegetative growth and to defense. In this study, the acceleration of flowering in response to ozone was particularly observed for the experiments that were performed in autumn, when flowering was overall delayed. Light quantity, quality and duration, as well as temperature, are usual cues that induce flowering (McClung et al., 2016). In the absence of these cues, stress factors may step in and act as flowering inducers (Takeno, 2016). Therefore, the lack of a strong light and/or temperature cue is possibly at the root of the acceleration of the onset of flowering by ozone observed in this study.

In the bumblebee experiment, there was a moderately higher number of flower visits to ozone-exposed plants than to control plants, which was driven by a higher number of open flowers in those plants. Flowering earlier may have conferred an advantage to ozone-exposed plants by reducing the competition in the beginning of flowering, when flowering plants were dominantly ozone-exposed plants. We expected that exposure of the plants to ozone would affect the attractiveness of the plants to pollinators, by altering the floral bouquet that is emitted by the plants and used by pollinators as olfactory cues. However, this does not seem to be the case. Recent work from Saunier and Blande (2019) assessed that wild mustard plants do not show effects of ozone, either on the floral volatile emission rates or on the floral blend composition. The plants may, however, have altered other floral traits in response to ozone, such as flower size, number of pollen grains, and nectar volume and concentration, as occurs with other stresses (Descamps et al., 2020; Rusman et al., 2019a; Rusman et al., 2019b). In fact, besides olfactory cues, pollinators are also attracted to flowers by visual cues (Chittka and Raine, 2006) and at least some pollinators favour visual cues over scent (Barragan-Fonseca et al., 2020). Therefore, an alternative explanation to the absence of effect of ozone on flower attractiveness is that in our experimental set-up, the use of visual cues was favoured in detriment of the chemical cues. Also, we should consider that the number of pollinators introduced in the greenhouse may have been too high, leading to a high demand/supply ratio for food resources. In a situation of low food supply, pollinators cannot afford to favour some plants over others, instead they forage to whatever they find.

The strongest effect of ozone in pollinator behaviour was the increase in the average time honeybees spent per flower. We hypothesize that this increase is due to changes in the quantity or quality of nectar produced by the flowers. Flowers of broad bean plants that grew under high ozone conditions and were transferred to ozone-clean conditions at flowering, produced a higher volume of nectar and this nectar had a higher concentration of sucrose and amino-acids compared to the flowers of plants that were permanently kept in ozone-clean conditions (Stabler, 2016). The fact that we only observed this effect in the honeybee experiment may be due to ozone x temperature interactions, since the honeybee experiment was performed in the warmest conditions and high temperatures were shown to have interactive effects with ozone (Duque et al., 2019). Alternatively, pollinator response to stress-induced plants may be species-specific, as shown in studies of *Brassica nigra* plants infested by herbivores (Bruinsma et al., 2014; Rusman et al., 2018), highlighting the importance of considering each pollinator separately.

Interestingly, ozone exposure only affected reproduction of the plants pollinated by the hoverflies. In the hoverfly experiment, there was a tendency for reduced total flower numbers, reduced vegetative plant mass, and reduced flower visitation in response to ozone. Although none of these effects was particularly important, collectively they lead to a reduction in the reproductive success rate of flowers, the number of fruits set and the total number of seeds produced by ozone-exposed plants, indicating that the effects of ozone accumulate. The observed reduced reproductive fitness may also come from decreased ovule viability following ozone exposure, as observed by Gillespie et al. (2015).

Conclusions

Reproduction is the ultimate goal of plants. In the presence of ozone, plants shifted their investment from vegetative growth to reproduction, by accelerating the onset of flowering. Exposure to ozone did not seem to have a strong effect on pollinator preference, but honeybees spent more time per flower on ozone-exposed plants, maybe due to higher nectar contents. Nevertheless, the plants' mechanisms to cope with ozone stress were not always enough to offset the effects of ozone: in one of our experiments, plants that had been exposed to ozone had decreased reproduction. Furthermore, it should be considered, that acceleration of flowering in a natural setting may lead to desynchronization between plant and pollinator activity and therefore to stronger indirect effects of ozone on plant reproduction than those observed here. Future studies on the effects of ozone on plant-pollinator interactions should take this into account. Additionally, attention should be directed to the interactive effects of ozone with other co-occurring global change drivers, such as increased temperature, increased drought episodes, and higher carbon dioxide concentrations, since they may either exacerbate or counterbalance the effects of ozone.

Supplementary material to chapter III

Table SIII.1 – Details on the periods the experiments were performed and the conditions in the ozone chamber during the fumigation period (ozone concentration, temperature and relative humidity (mean ± standard deviation), as well as accumulated ozone exposure (AOT40))

Experiment	Pollinator species	Round	Period of fumigation	[O3] ppb in the ozone chamber	AOT40 (ppb.h)	Temp (°C)	RH (%)	Period of visitation observations
1	*Osmia bicornis*	1	30/04 - 04/05/2019	114.62 ± 12.18	2238	27.5 ± 1.4	54.4 ± 4.1	05/05 - 11/05/2019
		2	07/05 - 11/05/2019	116.08 ± 7.34	2283	25.3 ± 4.5	48.0 ± 2.6	12/05 - 18/05/2019
2	*Apis mellifera*	1	28/05 - 01/06/2019	113.95 ± 13.71	2218	29.3 ± 1.6	51.7 ± 2.2	02/06 - 08/06/2019
		2	06/06 - 10/06/2019	120.07 ± 9.49	2402	29.5 ± 1.7	45.8 ± 2.2	11/06 - 17/06/2019
3	*Eristalis tenax*	1	16/09 - 20/09/2019	116.54 ± 7.31	2296	27.0 ± 1.1	44.2 ± 2.1	21/09 - 27/09/2019
		2	23/09 - 27/09/2019	116.28 ± 9.15	2289	29.1 ± 1.1	45.2 ± 2.8	28/09 - 04/10/2019
4	*Bombus terrestris*	1	07/10 - 11/10/2019	116.13 ± 7.99	2285	27.5 ± 1.3	55.5 ± 3.2	12/10 - 18/10/2019
		2	17/10 - 21/10/2019	114.09 ± 8.17	2223	28.4 ± 1.7	61.1 ± 4.3	22/10 - 28/10/2019

Fitted models

Model 1. Number of open flowers per plant ~ Treatment x Time + Treatment x Experiment + random (Experiment/Round)

Zero-inflation model: ~ Treatment x Time + Treatment x Experiment

Dispersion model: ~Time

Family = truncated_nbinom1

Model 2. Number of total flowers produced ~ Treatment x Experiment + random (Experiment/Round)

Family = nbinom2

Model 3. Plant vegetative mass ~ Treatment x Experiment + random (Experiment/Round)

Family = gaussian

Models 4. Number of flower visits performed per plant in 10 minutes (with and without offsetting for the absolute number of open flower and their relative numbers at any given period)

Model 4.1. Number of flower visits in 10 min ~Treatment x Time + random(Plant)

Zero-inflation: ~1

Family = nbinom1

Model 4.2. Number of flower visits in 10 min ~ Treatment x Time + offset(log(Number of open flowers)) + offset(Relative abundance of open flowers) + random(Plant)

Zero-inflation: ~1

Family = genpois

Models 5. Number of visitors received in 10 minutes (with and without offsetting for the absolute number of open flower and their relative numbers at any given period)

Model 5.1. Number of visitors received in 10 min ~Treatment x Time + random(Plant)

Zero-inflation:~1

Family = nbinom1

Model 5.2. Number of visitors received in 10 min ~Treatment x Time + offset(log(Number of open flowers)) + offset(Relative abundance of open flowers) + random(Plant)

Zero-inflation:~1

Family = genpois

Model 6. log(Average time spent per flower) ~Treatment x Time + random(Plant)

Family = gaussian

Model 7. Fruits produced ~ Treatment + Number of total flowers produced + random(Round)

Family = gaussian

Model 8. Success rate of the flowers ~ Treatment + random(Round)

Family = betabinomial

Model 9. Individual seed weight ~ Treatment + random(Round)

Family = gaussian

Model 10. Seeds produced per pod ~ Treatment + random (Round/Plant)

Family = gaussian

Model 11. log(Estimated total seeds produced) ~ Treatment + random(Round)

Family = gaussian

Model 12. Estimated total seed weight ~ Treatment + random(Round)

Family = gaussian

Results tables

Table SIII.2 – Effects of ozone on the number of open flowers during the 7 days following fumigation

	Conditional model			Zero-inflation model		
Explanatory variable	Chisq	Df	P-value	Chisq	Df	P-value
Treatment (Control vs Ozone)	1.9	1	0.165	13.1	1	**<0.001**
Time (Days after fumigation)	307.5	1	**<0.001**	60.9	1	**<0.001**
Experiment	43.7	3	**<0.001**	98.0	3	**<0.001**
Treatment x time	1.5	1	0.218	0.9	1	0.337
Treatment x Experiment	4.9	3	0.181	19.4	3	**<0.001**
	N = 896					

Table SIII.3 – Effect of ozone on the flowering onset within each experiment

Experiment	T-ratio	P-value
1	-0.481	0.631
2	-0.052	0.959
3	5.478	**< 0.001**
4	1.976	**0.049**

Table SIII.4 – Effects of ozone on the plants' reproductive and vegetative investment

	Number of total flowers produced			Plant vegetative mass		
Explanatory variable	Chisq	Df	P-value	Chisq	Df	P-value
Treatment (Control vs Ozone)	2.6	1	0.110	3.0	1	**0.084**
Experiment	121.2	3	**<0.001**	166.2	3	**<0.001**
Treatment x Experiment	1.0	3	0.813	4.0	3	0.256
	N = 107			N = 107		

Table SIII.5 – Effects of ozone on the number of flower visits a plant receives in 10-min observations by 4 different pollinators, without correcting for the amount of open flowers

	Apis mellifera			*Bombus terrestris*			*Eristalis tenax*			*Osmia bicornis*		
Explanatory variable	Chisq	Df	P-value	Chisq	Df	P-value	Chisq	Df	P-value	Chisq	Df	P-value
Treatment (Control vs Ozone)	0.8	1	0.373	2.9	1	**0.088**	0.9	1	0.333	1.5	1	0.222
Time (Days after fumigation)	52.4	1	**<0.001**	14.7	1	**<0.001**	4.9	1	**0.027**	0.8	1	0.365
Treatment x time	1.9	1	0.171	1.3	1	0.250	0.1	1	0.744	2.5	1	0.117
	N = 205			N = 112			N = 135			N = 178		

Table SIII.6 – Effects of ozone on the number of flower visits a plant receives in 10-min observations by 4 different pollinators, corrected for the amount of open flowers.

	Apis mellifera			*Bombus terrestris*			*Eristalis tenax*			*Osmia bicornis*		
Explanatory variable	Chisq	Df	P-value	Chisq	Df	P-value	Chisq	Df	P-value	Chisq	Df	P-value
Treatment (Control vs Ozone)	0.9	1	0.354	0.0	1	0.978	1.9	1	0.165	1.2	1	0.277
Time (Days after fumigation)	0.1	1	0.823	0.2	1	0.664	7.9	1	**0.005**	17.1	1	**<0.001**
Treatment x time	2.5	1	0.113	2.5	1	0.116	0.0	1	0.955	0.2	1	0.677
	N = 205			N = 112			N = 135			N = 178		

Table SIII.7 – Effects of ozone on the number of visitors a plant receives in 10-min observations during the week following fumigation (corrected for the amount of open flowers).

	Apis mellifera			*Bombus terrestris*			*Eristalis tenax*			*Osmia bicornis*		
Explanatory variable	Chisq	Df	P-value	Chisq	Df	P-value	Chisq	Df	P-value	Chisq	Df	P-value
Treatment (Control vs Ozone)	0.1	1	0.902	0.4	1	0.530	0.4	1	0.426	0.2	1	0.685
Time (Days after fumigation)	8.7	1	**0.003**	1.1	1	0.301	16.1	1	**<0.001**	15.1	1	**<0.001**
Treatment x time	3.2	1	**0.073**	0.6	1	0.433	0.2	1	0.640	0.0	1	0.981
	N = 205			N = 112			N = 135			N = 178		

Table SIII.8 – Effects of ozone on the number of visitors a plant receives in 10-min observations during the week following fumigation (not corrected for the amount of open flowers).

	Apis mellifera			*Bombus terrestris*			*Eristalis tenax*			*Osmia bicornis*		
Explanatory variable	Chisq	Df	P-value	Chisq	Df	P-value	Chisq	Df	P-value	Chisq	Df	P-value
Treatment (Control vs Ozone)	0.9	1	0.356	2.3	1	0.130	0.6	1	0.435	0.4	1	0.519
Time (Days after fumigation)	24.1	1	**<0.001**	14.6	1	**<0.001**	0.9	1	0.352	0.7	1	0.390
Treatment x time	2.9	1	0.090	0.6	1	0.423	1.6	1	0.210	0.9	1	0.330
	N = 205			N = 112			N = 135			N = 178		

Table SIII.9 – Effects of ozone on the average time a pollinator spends per flower

	Apis mellifera			*Bombus terrestris*			*Eristalis tenax*			*Osmia bicornis*		
Explanatory variable	Chisq	Df	P-value	Chisq	Df	P-value	Chisq	Df	P-value	Chisq	Df	P-value
Treatment (Control vs Ozone)	7.7	1	**0.010**	0.0	1	0.991	0.0	1	0.968	0.2	1	0.664
Time (Days after fumigation)	3.2	1	**0.073**	0.0	1	0.844	1.1	1	0.302	0.7	1	0.416
Treatment x time	0.0	1	0.943	0.7	1	0.393	0.1	1	0.764	0.6	1	0.440
	N = 300			N = 175			N = 105			N = 155		

Table SIII.10 – Effects of ozone on fruit set of the entire plant and separately for the terminal inflorescence

		Apis mellifera			*Bombus terrestris*			*Eristalis tenax*			*Osmia bicornis*		
	Explanatory variable	Chisq	Df	P-value	Chisq	Df	P-value	Chisq	Df	P-value	Chisq	Df	P-value
Plant	Treatment (Control vs Ozone)	1.7	1	0.191	0.5	1	0.478	4.8	1	0.029	1.2	1	0.269
	Number of total flowers	37.7	1	<0.001	76.2	1	<0.001	29.6	1	<0.001	28.4	1	<0.001
Terminal inflorescence	Treatment (Control vs Ozone)	0.2	1	0.679	0.3	1	0.581	6.9	1	0.009	1.0	1	0.320
	Number of total flowers	26.2	1	<0.001	14.12	1	<0.001	33.3	1	<0.001	103.9	1	<0.001
		N = 32			N=22			N = 23			N = 30		

Table SIII.11 – Effects of ozone on reproductive success rate, average seed weight, estimated total seed weight and estimated total number of seeds, for the entire plant and separately for the terminal inflorescence

		Apis mellifera			*Bombus terrestris*			*Eristalis tenax*			*Osmia bicornis*		
	Response variable	Chisq	Df	P-value	Chisq	Df	P-value	Chisq	Df	P-value	Chisq	Df	P-value
Plant	Reproductive success rate	1.9	1	0.165	0.8	1	0.370	2.8	1	**0.092**	0.3	1	0.602
	Average seed weight	8.7	1	**0.003**	2.7	1	0.103	1.6	1	0.207	0.5	1	0.476
	Estimated total seed weight	0.0	1	0.847	0.0	1	0.908	2.7	1	**0.099**	1.7	1	0.195
	Estimated total number of seeds	1.9	1	0.169	0.7	1	0.397	5.3	1	**0.021**	0.6	1	0.443
Terminal inflorescence	Reproductive success rate	0.0	1	0.973	0.5	1	0.473	5.2	1	**0.022**	0.3	1	0.571
	Average seed weight	3.2	1	0.075	1.1	1	0.286	0.0	1	0.967	1.3	1	0.253
	Estimated total seed weight	0.0	1	0.891	1.1	1	0.286	2.6	1	0.106	0.0	1	0.898
	Estimated total number of seeds	0.5	1	0.482	1.5	1	0.216	3.1	1	**0.079**	0.4	1	0.512
		N = 32			N = 22			N = 23			N = 30		

Table SIII.12 – Effects of ozone on the number of seeds produced per pod in the entire plant and separately for the terminal inflorescence

	Apis mellifera			*Bombus terrestris*			*Eristalis tenax*			*Osmia bicornis*		
	Chisq	Df	P-value	Chisq	Df	P-value	Chisq	Df	P-value	Chisq	Df	P-value
Plant	2.0	1	0.158	0.2	1	0.627	0.1	1	0.794	0.3	1	0.592
Terminal inflorescence	1.0	1	0.307	2.2	1	0.140	0.6	1	0.447	0.6	1	0.455
	N = 320			N = 220			N = 230			N = 300		

Supplementary figures

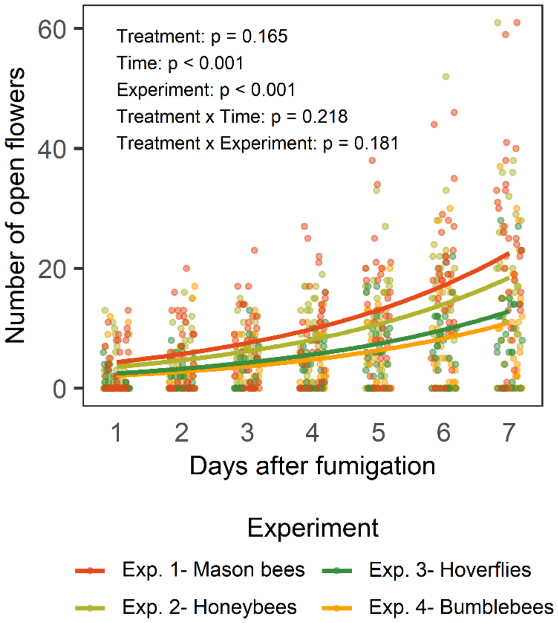


Figure SIII.1 – The flowering patterns of plants, in the 7 days after fumigation, differed between experiments. P-values are presented for all the fixed variables of the conditional model. The dots are the data points and the lines represent the regression model tested. Data points were jittered to improve visualization.

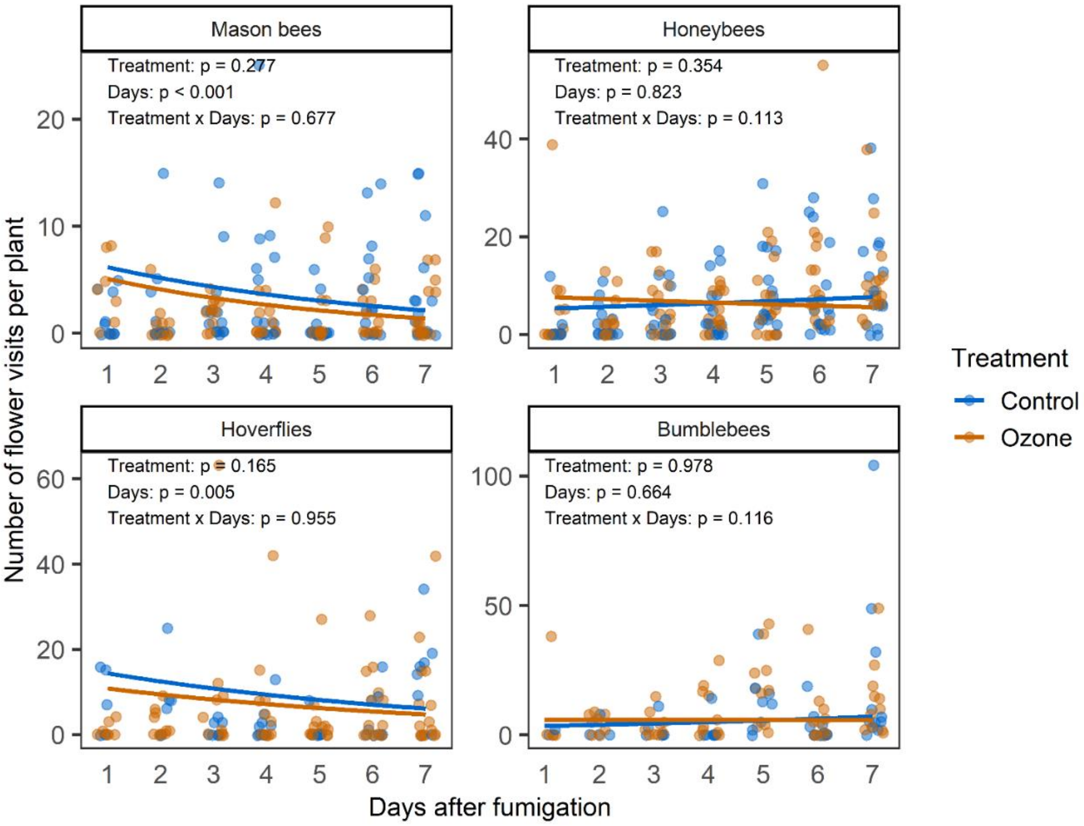
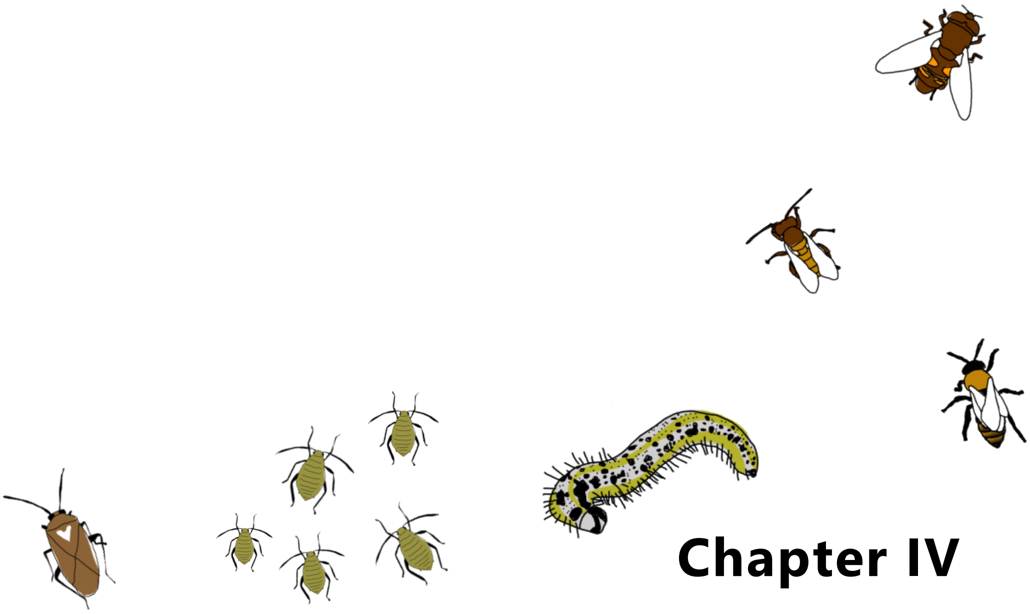


Figure SIII.2 – The effect of ozone on the number of flower visits a plant receives in 10-min periods on the 7 days following fumigation for four different pollinators. Models were corrected for the amount of open flowers available for pollination. The lines represent the regression lines of the models tested and the dots are the data points. Data points were jittered to improve visualization. P-values are presented for all fixed factors included in the model.



Chapter IV

Plant age at the time of ozone exposure affects flowering patterns, biotic interactions and reproduction of wild mustard

Exposure of plants to environmental stressors can modify their metabolism, interactions with other organisms and reproductive success. Tropospheric ozone is a source of plant stress. We investigated how an acute exposure to ozone at different times of plant development affects reproductive performance, as well as the flowering patterns and the interactions with pollinators and herbivores, of wild mustard plants. The number of open flowers was higher on plants exposed to ozone at earlier ages than on the respective controls, while plants exposed at later ages showed a tendency for decreased number of open flowers. The changes in the number of flowers provided a good explanation for the ozone-induced effects on reproductive performance and on pollinator visitation. Ozone exposure at earlier ages also led to either earlier or extended flowering periods. Moreover, ozone tended to increase herbivore abundance, with responses depending on herbivore taxa and the plant age at the time of ozone exposure. These results suggest that the effects of ozone exposure depend on the developmental stage of the plant, affecting the flowering patterns in different directions, with consequences for pollination and reproduction of annual crops and wild species.

This chapter is published as **Duque, L.**, Poelman, E.H., Steffan-Dewenter, I., **2021**. *Plant age at the time of ozone exposure affects flowering patterns, biotic interactions and reproduction of wild mustard*. Scientific Reports 11, 10.

Introduction

Air pollution is an anthropogenic driver affecting the health of both humans and terrestrial ecosystems (Agathokleous et al., 2020; Grulke and Heath, 2020; Manisalidis et al., 2020). Tropospheric ozone is a highly oxidative atmospheric pollutant that has the potential to change a plant's metabolism and therefore its interactions with other organisms (Agathokleous et al., 2020).

The formation and removal of tropospheric ozone is complex and it is in a constant balance that depends on pollutant emissions as well as climate and meteorology (Fitzky et al., 2019). Tropospheric ozone is a secondary pollutant, formed by the reaction of primary pollutants, namely nitrogen oxides (NOx) and volatile organic compounds in the presence of sunlight. It can be removed by NO (nitric oxide) titration, photolysis, deposition on surfaces and uptake by plants (Fitzky et al., 2019). Tropospheric ozone concentrations have greatly increased since pre-industrial times (Young et al., 2013). While the effort to reduce precursor emissions has led to a reduction of tropospheric ozone levels in some parts of the world (Lin et al., 2017; Yan et al., 2018), globally, ozone concentrations are still increasing (Ziemke et al., 2019), and in places like China, it is now an issue of major concern (Lu et al., 2020). Due to the highly complex balance of production and removal, ozone concentrations can increase even when air pollution in general decreases, as was the case during the first COVID-19 lockdown in some cities in Europe and China (Sicard et al., 2020) and can be higher away from the places where the majority of the primary pollutants are emitted, like rural areas (Fitzky et al., 2019; Yan et al., 2018), where it can affect vegetation.

In an ozone-polluted atmosphere, a plant's susceptibility and reaction to ozone may determine its competitiveness and therefore its persistence as part of a given plant community (Hayes et al., 2007; Martinez-Ghersa et al., 2017; Wedlich et al., 2012). Ozone pollution also alters root exudation, modifying soil microbial communities (Changey et al., 2018; Wang et al., 2017); it alters the nutritional value of plants, affecting the development of herbivores feeding on ozone exposed plant tissues (Couture and Lindroth, 2012; Couture et al., 2012; Duque et al., 2019; Khaling et al., 2015); and it changes plant volatiles, potentially altering plant attractiveness or repellence to insects (Giron-Calva et al., 2016, 2017; Li et al., 2016; McFrederick et al., 2009; Saunier and Blande, 2019).

Reproductive performance depends not only on the plant and its capability to react to a multitude of abiotic environmental factors (Dolferus et al., 2011; Leisner and Ainsworth, 2012; Zinta et al., 2016), but also on the interactions of the plant with other organisms in its ecosystem (Grass et al., 2018). Tropospheric ozone can reduce plant reproductive performance, as measured by the number of seeds produced by the plant, in a wide range of species (Leisner and Ainsworth, 2012; Singh et al., 2014). Some of the proposed mechanisms by which ozone might affect reproductive performance include 1) decreased photosynthesis by means of a) reduced photosynthetically active leaf area due to cell death or accelerated senescence or b) decreased Rubisco activity, 2) inhibition of assimilate translocation and 3) effects on reproductive processes such as decreased pollen germination, decreased pollen tube growth and abscission of reproductive sites (Fiscus et al., 2005; Leisner and Ainsworth, 2012). However, other factors should be taken into consideration, such as plant phenology, as well as interactions of plants with other organisms, namely their pollinators and herbivores. Ozone has been shown to alter the timing of the onset of flowering (Duque et al., 2021; Hayes et al., 2012; Rämö et al., 2007) and to decrease the number of flowers (Hayes et al., 2012; Rämö et al., 2007), which may alter reproductive success. For entomophilous plants (i.e. plants that depend mainly on insects for pollination) interaction with pollinators is key to their reproductive performance (Bommarco et al., 2012; Webber et al., 2020). Ozone can affect the floral volatiles that the plant emits to attract pollinators (Saunier and Blande, 2019), or posteriorly react with those volatiles in the atmosphere (Farre-Armengol et al., 2016). These changes in floral volatiles render the floral scent less attractive for naïve pollinators, but the pollinators may have the ability to learn these new scents (Cook et al., 2020a). However, few studies have addressed the subject of the impact of ozone on the interaction between plants and their pollinators from an empirical point of view. Another group of organisms that may strongly affect a plant's performance are herbivores, either by feeding on the plant vegetative parts and reducing plant photosynthesis, or by directly destroying reproductive organs or fruits. Most studies that assessed the effects of tropospheric ozone on plant-herbivore interactions have focused their attention on the effects of the modified interactions from the perspective of the herbivores (Couture and Lindroth, 2012; Couture et al., 2012; Duque et al., 2019; Khaling et al., 2015; Peltonen et al., 2010), while the consequences for plant reproduction are little explored. Since Lee et al. (1988) suggested that sensitivity of plants to ozone depended on the timing of exposure, a handful of studies assessed the effects of plant age/phenological stage during exposure on reproduction and other plant parameters (Lyons and Barnes, 1998; Pleijel et al., 1998; Reiling and Davison, 1992, 1994) and this factor was included in some ozone-exposure indexes (Mills et al., 2017). However, no single study tried to assess the effects of the timing of exposure to ozone on plant-insect interactions and the consequences for the reproductive performance of the plants.

In the present study, we exposed wild mustard (*Sinapis arvensis* L.) plants, in fumigation chambers, to an acute ozone exposure in four different periods of their life cycle. These plants were subsequently grown in a field where they were accessible for both pollinators and herbivores. We aimed to answer the following research questions: 1) Does an exposure to enhanced levels of ozone alter the reproductive performance of wild mustard plants and does this depend on the plant age at the time of exposure? 2) When reproductive performance is affected, is this due to compensatory responses in the plant’s phenology? 3) Is this due to altered interactions between the plant and its pollinators? 4) Is this due to altered interactions between the plant and its herbivores?

Material and methods

This experiment was performed between April and October 2017. Wild mustard (*Sinapis arvensis* L.) plants were exposed to a target level of 120 ppb of ozone in a fumigation system placed in a greenhouse. Plants were exposed at different ages (Fig. IV.1). Following exposure, plants were placed outdoors to be openly exposed to pollinators and herbivores. Plant reproductive performance was assessed. All methods were performed in accordance with the relevant guidelines and regulations.

Plant material

Native to Europe, the Middle East and Western Asia, wild mustard is now widespread through most of the temperate regions of the world, where it is considered a weed (Warwick et al., 2000). It is an annual entomophilous plant with an indeterminate growth habit (i.e. it continues to produce flowers when the reproductive success is low) (Mulligan and Bailey, 1975; Warwick et al., 2000). As a plant whose germination starts mainly in early spring (Fogg, 1950), wild mustard is at risk of increased ozone exposure, as the timing of maximum exposure to ozone is shifting from Summer to Spring (Neufeld et al., 2019).

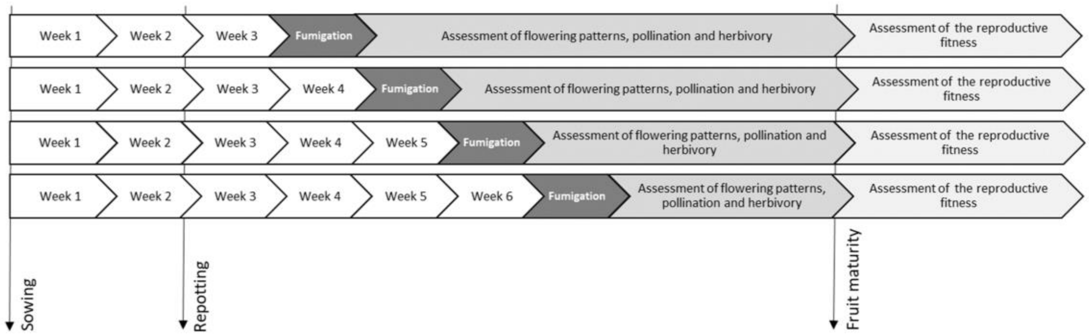


Figure IV.1 – Chronology of the experiment

For this study, wild mustard seeds were provided by the Botanical Garden of Konstanz, Germany. The seeds were sown in seed trays and repotted 2 weeks later into 18 x 18 cm pots. A 2:1-mixture of peat based substrate (Einheits Erde CL ED 73) and sand (Hamann Filtersand 0,7-1,25 mm) was used both in the seed trays and in the pots. The plants were watered throughout the experiment according to their needs and no fertilizer was added. 3 cohorts of plants were sown on the 26th April, 14th June and 12th July 2017. 32 plants were used per cohort. The 1st cohort was kept in a greenhouse at all times until after fumigation. This cohort of plants was found to be very tender, breaking easily when put outside after fumigation. Therefore, for the 2nd and 3rd cohorts, the plants were often taken outside of the greenhouse before fumigation, during daylight hours, for hardening off.

To test whether the effects of ozone are dependent on plant age at the time of fumigation, plants were fumigated at different ages, with plant age being counted from the day of sowing. Hence, each week since the plants were 3 weeks old up until when they were 6 weeks old, 8 plants (4 per treatment level) were chosen for fumigation. Plant selection and assignment of fumigation treatment were random, except that it was made sure all plants in one round of fumigation were in the same phenological stage. Plants from the first cohort developed more slowly, presumably due to the meteorological conditions observed, therefore plant age and plant phenological stage at the beginning of the fumigation treatment are mostly overlapping but do not totally coincide for all cohorts (Table SIV.1).

Ozone fumigation

Fumigation took place in a fumigation system with 2 glass chambers placed in a greenhouse (Duque et al., 2019). In this fumigation system, the air is supplied by a compressed air system and passes through an activated charcoal filter and a particle filter before it reaches the chambers. To increase the ozone level, a small portion of the incoming air diverges from this main stream and passes through an air dryer (AIRdyer3.1, INNOTEC) and a customized ozone generator (INNOTEC high engineering GmbH) before it reaches one of the chambers. To attain the desired ozone-enhancement, the ozone generator is regulated by a controller connected to an ozone analyser (APOA-370, Horiba Ltd). The air comes into the chambers through an opening at the top of the door frames and leaves the chambers passively through an opening at the bottom of the door frames. The air that is analyzed derives from two different points at different heights in the chambers. When the air is not being ozone-enhanced, the ozone concentration is below 1 ppb.

The fumigation treatment consisted of exposing plants to 2 different levels of ozone: ozone-clean and ozone-enhanced. The chambers were randomly assigned an ozone level before each fumigation round. The ozone-enhanced level (henceforward called ozone) consisted of exposing the plants to a target ozone concentration of 120 ppb of ozone for 6 hours/day from 11:00 to 17:00 CEST and to < 1 ppb of ozone for the rest of the day. Due to some technical problems, the ozone concentration during the fumigation was very variable, particularly for the first plant cohort (table S1). In the ozone-clean level (henceforward called control) the plants were exposed to < 1 ppb of ozone all day long. The fumigation treatments lasted 7 consecutive days.

120 ppb ozone is a very high tropospheric ozone concentration, but one that is sometimes exceeded during high ozone episodes in the current times and is expected to occur more often in the future in the developing regions of the world (Lei et al., 2012). However, these exact conditions are unlikely to occur, as in nature, ozone concentration will not rise from < 1 ppb to ~ 120 ppb in a matter of minutes or drop again to < 1 ppb in a short period. On the other hand, the control treatment

does not represent the ambient ozone levels currently observed in the troposphere, but the absence of ozone.

Pollination and herbivory

At the end of each fumigation, the plants were placed outside in an experimental garden. The plants were kept in the pots and the pots were ~80% buried in the ground. The plants from each cohort were randomly positioned in an 8-row rectangular design with 2 m distance between plants.

During flowering, plants were observed for 4-min periods, ~3 times per week, for flower visits. We did 19 ± 5 observations per plant for a total of 1588 4-min observations. The sequence of plants observed on each day was randomized. During these observations, we recorded the number of flower visitors, the visitor guild (wild bee, honeybee, bumblebee, syrphid fly, other flies, butterflies and others), and, when possible, the number of flowers visited. Ants and rape pollen beetles were also found on the flowers, but they were disregarded, for our purpose was to assess possible pollinator visitation and they are not expected to provide pollination services. In more than 90% of the observation periods, the number of open flowers per plant was also counted. Missing data on the number of open flowers was predicted by fitting a flowering curve for each plant using the gam function (mgcv package version 1.8-31 (Wood, 2003)) in R .

The plants were observed 1-2 times per week for the presence of herbivores and predators (abundance) or signs of herbivory (percentage of leaf tissue damaged by chewing herbivores). When abundance of herbivores was above circa 200 (this was the case for aphids only), abundance was estimated by considering the area occupied by a subset of herbivores. Percentage of leaf tissue damaged by chewing herbivores, henceforward referred to as herbivore damage, did not include leaf mining.

Reproductive success

At fruit maturity, the number of seeds produced per pod was counted for a set of 20 pods per plant (when possible) and 100 seeds per plant (when possible) were weighed. The plants were collected and the total number of fruits produced per plant, as well as the total number of reproductive sites that did not set fruit, were counted. Using the data collected, we estimated the total number of seeds (= number of fruits produced x average number of seeds per pod) and the total seed weight (= estimated total number of seeds x average weight of one seed) produced by the plant. The 10 plants that produced an estimated number of seeds lower than 100 were not considered in the statistical analysis of this study, because we considered they had an abnormal development (number of seeds per plant was 4778 ± 2477 (mean ± sd)).

Statistical analysis

All statistical analyses were performed in R version 4.0.1 (R Core Team, 2020). To answer our research questions, we fitted (generalized) mixed effects models using the glmmTMB function from the glmmTMB package version 1.0.1 (Brooks et al., 2017). The fit of the models was tested using the Dharma package version 0.3.1 (Hartig, 2020). For all models, we used the factors treatment (control vs ozone), plant age at the time of exposure and their interactions as fixed factors and plant cohort as a random factor. Other variables were only included when they improved the fit of the models (see table S2 for further details on the models). Furthermore, we used AICc (Akaike Information Criterion, corrected for small sample sizes) to select the best model predicting the number of seeds produced per plant. Post-hoc tests were performed to assess at what plant age exposure to ozone is relevant. Post-hoc tests as well as model predictions presented in the graphs, were obtained using the emmeans and emtrends functions in the emmeans package version 1.4.7 (Lenth, 2020a).

Results

Wild mustard plants were affected by ozone differently in their reproductive performance depending on the age of the plant at the beginning of the fumigation period (Treatment x Plant age: $p = 0.006$, $p < 0.001$ and $p = 0.003$, for the number of fruits, number of seeds and total seed weight produced, respectively, Fig. IV.2, Table SIV.3). Reproductive performance of control plants differed between plants exposed at different ages likely due to the different amounts of time they were kept in the more favorable greenhouse conditions. In 3-week old plants, ozone exposure improved the reproductive performance of plants: they produced 1.7 times more fruits, 2.4 times more seeds and 2.4 times higher total seed weight than the respective control plants (p = 0.004, 0.002 and 0.006, respectively, Table SIV.3). The plants showed a tendency to have reduced reproductive performance when fumigated at later ages, particularly when fumigated at age 5 and 6 weeks. This tendency was only significant for the total seed weight of plants exposed at 5 weeks, when the yield of ozone-exposed plants was only 58 % of the control plants (p = 0.035). The results from flowering phenology, plant-pollinator and plant-herbivore interactions provide possible explanations for these plant age-dependent responses to ozone stress.

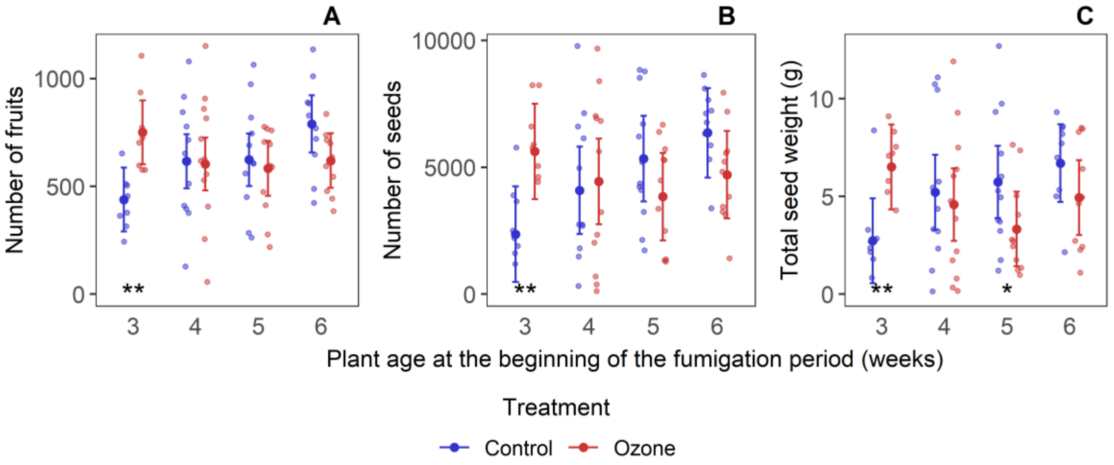


Figure IV.2 – The effect of exposure to ozone at different plant ages on the reproductive performance of individual plants. The dots are data points and the error bars represent the means and standard deviations as provided by the models tested. The asterisks represent significant effects of ozone exposure within plant age (* 0.05 ≥ p ≥ 0.01; ** 0.01 > p ≥ 0.001).

Flowering phenology

Ozone exposure affected the flowering patterns of the plants differently according to plant age (Treatment x Plant age, p = 0.027; Treatment x Plant age x DAS (Days after sowing), p < 0.001, Fig. IV.3, Table SIV.4). Plants that were exposed to ozone when they were 3 weeks old had, on average, more open flowers on the observation days than control plants (p = 0.008, Table SIV.5). In addition, exposure to ozone modified the flowering curve of plants exposed when they were 3 and 4-weeks old (p < 0.001, Fig. IV.3, Table SIV.5). Ozone exposure led to earlier flowering of plants exposed at 3 weeks, while plants exposed when they were 4 weeks old started flowering at the same time as control plants but reached peak flowering later and prolonged the flowering period (Fig. IV.3). There was no significant effect of ozone on the flowering patterns of plants fumigated at later ages, but we observed a tendency for a decrease in the number of open flowers ($p = 0.285$ and $p = 0.062$ for plants fumigated at ages 5 and 6 weeks respectively, Table SIV.5, Fig. IV.3).

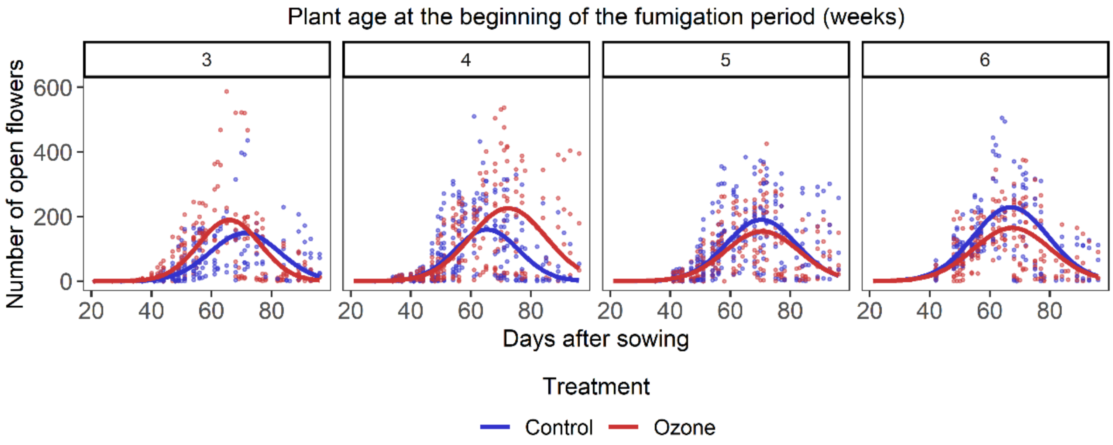


Figure IV.3 – The effect of exposure to ozone at different plant ages on the flowering patterns of individual plants. The dots are data points and the curves are the regression lines as provided by the model.

Plant-pollinator interactions

Of the registered 3150 flower visits, 90% were performed by bees and syrphids (69 and 20%, respectively). The other flower visitors were flies (7%), butterflies (1%) and other insects (2%). Amongst bees, 66% were honeybees and 34% were wild bees, while the representation of bumblebees was lower than 1%. The response of the flower visitors to the ozone treatment depended on the plant age at the beginning of the fumigation period (Treatment x Plant age: $p < 0.001$, Table SIV.6). In general, there was a tendency for plants that were fumigated with ozone at earlier ages to have more flower visits than the respective controls, while this tendency was reverted for plants fumigated at later stages (Table SIV.6 and Fig. SIV.1). However, the response varies with the pollinator guild considered, with bees showing this pattern more clearly than syrphids or the overall visitors (Table SIV.6 and Fig. SIV.1). When refitting the models tested by correcting for the number of open flowers, no effects of ozone on the number of visitors were observed, except for the group of large syrphids, that still showed a positive effect of ozone exposure at the 3 weeks stage on flower visitation ($p = 0.021$, Table SIV.7). This indicates that the effects of ozone on the number of flower visitors were mainly due to the changes in the number of open flowers. The number of flowers visited by a single visitor in a 4-minute interval was not affected by ozone (Treatment: $p = 0.726$), independently of the timing of exposure to ozone (Treatment x Plant age: $p = 0.462$).

Plant-herbivore interactions

Several herbivores and predators were observed on the plants after they were placed in the field. Although we often observed butterfly eggs of the species *Pieris rapae* and *Pieris brassicae*, we rarely saw any caterpillars, indicating high predation rates or plant resistance to insect eggs. The most abundant herbivores were aphids, especially of the species *Brevicorine brassicae* and *Myzus persicae*. Other insects that we observed in greater numbers on the plants included *Lygus pratensis* bugs and larvae of the aphid midget *Aphidoletes aphidimyza*. Although ozone did not have a strong effect on the level of damage by chewing herbivores (Treatment: $p = 0.127$, Treatment x Plant age: $p = 0.263$, Table SIV.8), the damage tended to be higher on plants fumigated with ozone at plant age 6 weeks than on control plants ($p = 0.015$, Table SIV.8, Fig. SIV.2). Also, on the rare occasions when ozone had an effect on insect abundance, the effect was positive for herbivores and negative for predators (Table SIV.8, Fig. SIV.2). The number of aphids was higher on plants fumigated with ozone at plant age 4 weeks ($p = 0.041$), the number of *Lygus pratensis* bugs was higher on plants fumigated with ozone at plant age 3 weeks ($p = 0.049$), but the number of aphid midget larva was lower on plants fumigated with ozone at plant age 6 weeks ($p = 0.015$), than on the respective controls.

Including herbivory (average number of aphids and average herbivore damage), pollination (average visitation rate) and average number of open flowers as predictor variables in the model predicting the number of seeds produced by the plants improved the model fit, while adding only the average number of open flowers provided the best model (Table SIV.9). The number of seeds produced by the plant increased with increased number of open flowers on the plants ($p < 0.001$,

Table SIV.9, Fig. IV.4). Including the average number of open flowers in the model resulted in a weaker pattern of the direct effects of ozone on the number of seeds produced (Treatment x Plant age: p = 0.018, Table SIV.9), while still showing a strong positive effect of ozone exposure on plants fumigated when they were 3 weeks old (p = 0.003, Table SIV.9, Fig. IV.4). This indicates positive effects of ozone on the number of seeds produced that go beyond the increase in the number of flowers.

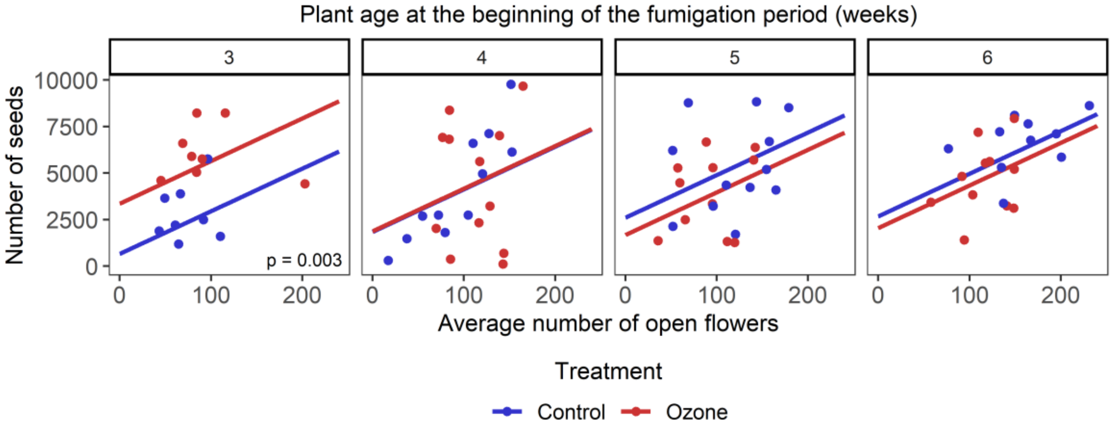


Figure IV.4 – The effect of ozone exposure at different plant ages on the number of seeds produced by the plants in relation to the average number of open flowers recorded during observations days. The lines represent the best model tested (lowest AICc) for predicting the number of seeds. Regression lines are provided by the emmeans package. P-values are presented for the effect of ozone when p ≤ 0.05.

Discussion

Subjecting wild mustard plants to an acute ozone exposure at different stages of their life revealed that ozone had opposite effects on plant reproductive performance depending on plant age at the time of exposure. While younger plants tended to overcompensate, meaning they responded to ozone stress by increasing reproductive performance, older plants were less plastic in their responses and tended to show reduced reproductive performance. By analyzing possible underlying mechanisms that led to these differences, we could reveal that 1) the effects of ozone on the flowering patterns of the plants depended on plant age during exposure, 2) the number of pollinators visiting the plants depended on plant age during exposure and was mostly driven by the different number of flowers open for pollination, 3) ozone stress tended to increase herbivory, particularly the abundance of sucking herbivores, but variation among taxa was large, 4) the higher reproductive performance of plants exposed in early ages was mostly driven by increased production of flowers.

Plant reproduction was affected by ozone, but the direction of the effects depended on the age of the plants at the time of exposure. Number of fruits, number of seeds and total seed weight produced by the plants was higher on plants exposed to ozone when they were 3 weeks old than on the respective controls, while exposing plants to ozone later in their life cycle tended to have the opposite effect. Studies reporting positive effects of acute ozone exposure on plant reproduction are uncommon (Leisner and Ainsworth, 2012). Previous studies that assessed the influence of plant age/developmental stage on the effects of ozone on *Plantago major* showed only negative effects of ozone on reproduction and only when exposure occurred during the early stages of flowering (Lyons and Barnes, 1998; Pleijel et al., 1998). Here, we observed a reduction of total seed weight of plants that were exposed to ozone when they were 5 weeks old. These plants were in the inflorescence emergence stage at the beginning of fumigation, but before the end of the fumigation period most of the plants had started flowering. This shows that plant reproductive sensitivity to ozone is the highest in the beginning of flowering also in wild mustard. However, it is possible that the age-dependent effects of ozone on plant reproduction are not only related to plant phenological stage during exposure but also to the changing conditions in the canopy as plants grow. We registered an increase in mean air relative humidity in the fumigation chambers as plants were fumigated at increasingly older ages (Table SIV.1). This is likely a result of increased transpiration due to increased leaf area index (Hardwick et al., 2015), which in turn is related to plant growth/age. In conditions of increased relative humidity, plants tend to open the stomata, increasing stomatal conductance and therefore ozone uptake (Gong et al., 2021). Increased ozone uptake is associated with stronger negative effects of ozone on vegetation (Emberson et al., 2018). Thus, ozone uptake would be higher when plants are fumigated at older ages, which would explain the tendency for negative effects of ozone on the reproduction of plants fumigated at older ages.

We further investigated what could be at the origin of the observed differences in the response of plants exposed to ozone at different plant ages on reproduction. Analyzing the flowering patterns of the plants, we observed that plants exposed to ozone when they were 3 weeks old had more flowers than the respective controls, with a tendency for the direction of these effects to be reverted as the plants were exposed at progressively older ages. Previous studies that assessed the number of flowers of plants exposed to ozone showed that this pollutant either did not change or reduced the number of flowers (Hayes et al., 2012; Rämö et al., 2007). In the light of our results, this could be due to the fact that these observations were made on older plants. However, the ozone fumigation in the referred studies corresponded to a long-term exposure of the plants and the plants used were perennials, which do not necessarily show the same sensitivity to ozone as annuals like wild mustard (Hayes et al., 2007; Leisner and Ainsworth, 2012). Also, having an indeterminate growth habit, wild mustard may have higher chances of compensating for negative effects of abiotic stresses than determinate plant species (Black et al., 2000), although reproductive growth habit alone does not explain the species-specific effects of ozone on the number of flowers (Leisner and Ainsworth, 2012). In our study, 3-week-old exposed plants started flowering earlier when fumigated with ozone, while 4-week-old exposed plants showed prolonged flowering. In a previous study we had already observed that ozone promoted earlier onset of flowering in wild mustard (Duque et al., 2021). Although in that study the fumigation was performed when the plants were 4 weeks old, on average they were likely in an earlier stage in their life cycle than in the present study due to the observed conditions during their development. Earlier flowering onset is a common phenomenon when plants are under abiotic stresses, such as poor nutrition, drought, high salinity and high or low temperatures (Takeno, 2016), but it can also be observed on plants exposed to biotic stresses, such as herbivory and damage caused by pollinators (Pashalidou et al., 2020; Pashalidou et al., 2013). Together, our results suggest that compensatory stress responses of young wild mustard plants in the ozone treatment lead to higher investment in flower production, together with earlier flowering or extended flowering times. In contrast, stress responses of more mature annual plants and of perennial plants are less plastic and cannot compensate for negative effects of ozone stress.

In our experiment, we were also interested in assessing other indirect effects of ozone stress on plant reproductive performance, namely those related to mutualistic and antagonistic plant-insect interactions. The ozone fumigation and the timing of the exposure affected the number of flower visitors on the plants. Although the results depended on the pollinator guild considered, there was an overall tendency for positive effects of ozone on the number of visitors received by plants exposed at younger ages (3- and 4-week-old), but negative effects on plants exposed at older ages (5- and 6-week-old). When correcting for the number of open flowers, these effects were no longer detectable, suggesting that the changes in the number of flower visitors was driven, in this experiment, by the change in the number of open flowers (Duque et al., 2021). In the present study, an exception was observed for the number of large syrphid flies visiting the plants, which was still higher on plants exposed to ozone when they were 3 weeks old than on the respective controls after the correction for the number of open flowers. These large syrphid flies possibly used other floral traits besides flower number as a cue for the quantity or quality of the rewards available in the plants. Stabler (2016) showed that exposure of broad bean plants to ozone reduced the amount of pollen produced and decreased its protein content, while the production of floral nectar was increased by short-term exposures and the nectar had a higher concentration of sucrose and amino acids when the exposure was interrupted at flowering. Pollinator requirements are species-specific. Unlike bees, that need to provide their offspring with enough resources to sustain them through the larval stage and, therefore, need to forage for higher quantities of protein in the form of pollen, syrphid flies, and especially large syrphid flies, consume pollen mainly for the maturation of the reproductive organs, therefore foraging mostly for nectar, their source of energy to fuel flight and increase longevity (Cook et al., 2020b). Therefore, we consider that the possible effects of ozone on the quantity and quality of nectar may have served as attractants to this group of foragers, possibly by encouraging revisits of previously visited plants. While ozone was shown to change the profile of floral scents emitted by the plants, this effect seems to be absent in wild mustard, that maintains both its floral volatile blend composition and rate of emission (Saunier and Blande, 2019), which might explain the absence of stronger plant-mediated effects of ozone on flower visitation.

The plant-mediated effects of ozone on herbivory were less clear than those on flower visitation, but in the few situations where effects were observed, they were positive, meaning ozone increased herbivory in exposed plants. This seems to contradict most studies on effects of ozone on the oviposition preference of herbivores, which report a reduced preference for ozone-exposed plants (Cui et al., 2014; Duque et al., 2019; Jones and Coleman, 1988; Kopper and Lindroth, 2003).

However, several studies refer to a reduction in the nutritional quality of leaves of plants exposed to elevated levels of ozone (Couture and Lindroth, 2012; Couture et al., 2012; Khaling et al., 2015), which may lead to compensatory feeding mechanisms, while other studies suggest an increase in leaf palatability (Abu ElEla et al., 2018). In both cases there may be an increase in consumption of leaf material (Abu ElEla et al., 2018; Bolsinger et al., 1992; Freiwald et al., 2008; Jones and Coleman, 1988; Khaling et al., 2015), therefore increasing the damage by chewing herbivores, a general tendency observed in this study that was only significant for plants exposed to ozone at age 6-weeks. The plant-mediated effects of ozone on the preference and performance of aphids are more ambiguous and depend on the level of exposure and the age of the exposed plants (Holopainen, 2002). The latter was the case in our study, with higher numbers of aphids on plants exposed when they were 4 weeks old than the respective controls, but no difference being observed when plants were exposed to ozone at other ages. Holopainen and Kössi (1998) observed that ozone can stimulate aphid population growth of aphids feeding on spruce seedlings exposed to 80 ppb of ozone, but aphids are not affected when the host plants are exposed to other levels of ozone or when the exposure continues until shoot maturity. Other studies showed that aphids tolerate well the plant-mediated effects of ozone and are not affected by the exposure(Manninen et al., 2000; Peltonen et al., 2006; Telesnicki et al., 2018). We also observed that the number of *Lygus pratensis* bugs was higher on plants exposed to ozone when they were 3 weeks old. This contrasts with the results obtained by Manninen et al. (2000) for another *Lygus* species (*Lygus rugulipennis*), whose performance was reduced in ozone-exposed Scots pine seedlings. Except for the study of Telesnicki et al. (2018) that used *Triticum aestivum* as the plant species, all other studies mentioned above on sucking herbivores were performed with perennial plant species, meaning that the longevity of the plant may influence its ozone plant-mediated effects on the performance of the herbivores.

On the other hand, predation, as measured in this study by the number of aphid midget larvae, followed the opposite trend of herbivory, with lower numbers of these larvae being observed on plants exposed to ozone at plant age 6-weeks than on the respective controls. Vuorinen et al. (2004) did not observe any changes in attraction of predatory mites following plant exposure to ozone, since ozone-exposed plants emitted volatiles were similar to those of plants infested with spider mites. Studies on the effects of ozone on multitrophic interactions focused mostly on parasitism of insect herbivores and the potential of ozone to disrupt the attraction of parasitoids due to chemical reactions with signaling volatiles in the atmosphere (Khaling et al., 2016; Pinto et al., 2007), or the potential of ozone to lead to plant volatiles emissions that are similar to those of herbivore-infested plants (Cui et al., 2014). Studies on the plant-mediated effects of ozone on insect herbivore predation are, however, largely missing and require further investment.

Including flowering, pollination and herbivory as potential co-variables in the model predicting the number of seeds produced per plant demonstrated that the number of flowers per plant is a key factor that shows indirect effects of ozone. Furthermore, the increase in the number of open flowers that results from exposing the plants to ozone at early ages is one way the plant reallocates its resources to reproduction.

In nature, it is possible that plants will experience high ozone levels at several points during their life cycle. Future studies should contemplate this hypothesis and try to assess whether the flower-stimulating effects observed in plants exposed at early ages would be counterbalanced when the plants would be exposed to a second ozone episode later in their lives. Also, studies in a more natural setting and studies including a wider variety of plant exposures to ozone (acute vs chronic) should be performed in order to assess if comparable results are observed.

In this study, we used a stream of compressed air as the feed gas for the ozone generator. Silent discharge ozone generators produce small amounts of nitrogen oxides when using dry air as the feed gas, particularly N_2O_5 (dinitrogen pentoxide) (Kogelschatz and Eliasson, 2018). It could be argued that this way of generating ozone produces confounding effects, making it hard to disentangle whether the effects observed are related to the increase in ozone concentration or to unwanted by-products of the ozone generation. However, the increase in concentration of these by-products is kept two orders of magnitude below the ozone concentration (Kogelschatz and Eliasson, 2018). Furthermore, there is little evidence that the amount of N_2O_5 produced would have strong effects on plants' development and growth, although some effects on the amounts of leachable nitrate and some other ions have been observed in Norway spruce needles when using ozone concentrations 2.5 times higher than the one used in our study (Brown and Roberts, 1988). However, to avoid the uncertainty of whether ozone generation by-products are interfering in the results, future studies should use pure oxygen as the feed gas in silent discharge ozone generators.

Conclusions

Overall, our study shows that, along with ozone exposure levels, conditions during exposure and susceptibility of plants, the plant age/ plant phenological stage at the time of exposure is also key to understanding the effects of ozone on reproductive performance. Plant age does not only affect the susceptibility of the plants but also the direction of the effects of ozone. In this study, an acute exposure to ozone at an earlier age resulted in higher reproductive performance of wild mustard plants while plants exposed later showed a tendency for the opposite effect. Also, the changes in the number of flowers provided a good explanation for both the changes in reproductive performance and the changes in pollinator visitation.

Supplementary material to chapter IV

Table SIV.1 – Plant phenological stage at the beginning of each fumigation round and conditions in the ozone chamber between 11h00 and 17h00, during the treatment days, for each fumigation round: average ozone concentration, accumulated exposure to ozone (AOT40), air temperature and relative humidity.

Plant cohort	Plant age	Plant phenological stage	$[O_3]$ in the ozone chamber (ppb)	AOT40 (ppb.h)	Air temperature (°C)	Air relative humidity (%)
1	3	Vegetative	95.5 ± 45.0	1872	28.6±1.9	46.0±2.4
	4	Vegetative	97.5 ± 40.5	1530	30.6±2.4	53.4±1.6
	5	Inflorescence emergence	68.9 ± 56.1	791	29.8±1.9	55.6±2.9
	6	Flowering	89.7 ± 44.9	1361	28.0±1.7	69.0±2.9
2	3	Vegetative	113.9 ± 11.9	2277	31.8±2.0	43.7±2.0
	4	Inflorescence emergence	112.2 ± 21.2	2227	29.7±2.5	49.0±2.2
	5	Inflorescence emergence	117.1 ± 10.0	2309	29.6±3.0	57.2±4.3
	6	Flowering	116.7 ± 9.5	2298	29.4±2.5	58.5±4.3
3	3	Vegetative	116.3 ± 11.4	2261	31.4±2.3	41.4±1.5
	4	Inflorescence emergence	117.1 ± 7.0	2310	28.7±2.1	48.6±1.7
	5	Inflorescence emergence	113.9 ± 7.9	2229	28.7±2.3	62.1±2.8
	6	Flowering	116.0 ± 8.7	2271	30.7±2.0	62.2±3.1

Table SIV.2 – Details on the models tested.

Response variable	Predictor variables	Random factors	Overdispersion factors	Zero-inflation factors	Family	Comments
Number of fruits	Treatment Plant age Number of reproductive sites Treatment x Plant age	Plant cohort	----	----	Gaussian	
Estimated number of seeds	Treatment Plant age Treatment x Plant age	Plant cohort	----	----	Gaussian	Mean number of open flowers, mean number of aphids, mean herbivore damage and mean visitation rate were included as possible predictors
Estimated total seed weight	Treatment Plant age Treatment x Plant age	Plant cohort	----	----	Gaussian	
Number of open flowers	Treatment Plant age poly (DAS,2) Treatment x Plant age Treatment x poly (DAS,2) Plant age x poly (DAS,2) Treatment x Plant age x poly(DAS,2)	DAS|Cohort Plant	Treatment Plant age poly (DAS,2) Treatment x Plant age Plant age x poly (DAS,2)	----	Negative binomial 1	
Number of flower visitors	Treatment Plant age Treatment x Plant age	Cohort/DAS Plant	1	1	Negative binomial 1	Models ran with and without offset term (log(Number of open flowers))

Table SIV.2 (continued) – Details on the models tested.

Response variable	Predictor variables	Random factors	Overdispersion factors	Zero-inflation factors	Family	Comments
Number of flowers visited	Treatment Plant age Treatment x Plant age	Cohort/DAS Plant	Number of open flowers	----	Negative binomial 2	
Level of chewing-herbivore damage	Treatment Plant age poly(DAS,2) Treatment x Plant age	DAS|Cohort Plant	Plant age poly (DAS,2)	poly(DAS,2)	Gaussian	
Number of aphids	Treatment Plant age poly(DAS,2) Treatment x Plant age Plant age x poly(DAS,2)	DAS|Cohort Plant	Plant age poly(DAS,2)	1	Generalized poisson	
Number of Lygus pratensis	Treatment Plant age DAS Treatment x Plant age	DAS|Cohort Plant	DAS	1	Negative binomial 2	
Number of aphid midget larva	Treatment Plant age poly(DAS,2) Number of aphids Treatment x Plant age	DAS|Cohort Plant	----	1	Poisson	

DAS - Days After Sowing. Poly(DAS,2) indicates that DAS is a quadratic term in the model. Cohort/DAS specifies variable means for each day within each cohort. DAS|Cohort specifies variable slopes for each cohort

Table SIV.3 – The effect of ozone exposure at different plant ages on the reproductive performance of the plants. Cells shaded in green and red represent positive and negative effects of ozone, respectively.

Response variable	Anova (type II)				Pairwise post-hoc (Control - Ozone)		
	Predictor variable	Chisq	df	p-value	Plant age	t-ratio	p-value
Number of fruits produced	Treatment	0.00	1	0.951	3	-2.97	**0.004**
	Plant age	3.41	3	0.332	4	0.14	0.889
	Number of reproductive sites	3.66	1	0.056	5	0.45	0.652
	Treatment x Plant age	12.47	3	**0.006**	6	1.86	0.067
Estimated number of seeds produced	Treatment	0.06	1	0.811	3	-3.31	**0.002**
	Plant age	6.46	3	0.091	4	-0.42	0.676
	Treatment x Plant age	18.01	3	**<0.001**	5	1.82	0.073
					6	1.90	0.061
Estimated total seed weight	Treatment	0.90	1	0.343	3	-2.82	**0.006**
	Plant age	2.76	3	0.429	4	0.57	0.572
	Treatment x Plant age	14.25	3	**0.003**	5	2.15	**0.035**
					6	1.50	0.138

Table SIV.4 – The effect of ozone exposure at different plant ages on the flowering patterns of the plants. DAS stands for Days After Sowing.

Response variable	Anova (type II)			
	Predictor variable	Chisq	df	p-value
Number of open flowers	Treatment	0.06	1	0.799
	Plant age	19.04	3	**< 0.001**
	poly (DAS,2)	2069.43	2	**< 0.001**
	Treatment x Plant age	9.18	3	**0.027**
	Treatment x poly (DAS,2)	0.16	2	0.921
	Plant age x poly (DAS,2)	55.43	6	**< 0.001**
	Treatment x Plant age x poly(DAS,2)	83.85	6	**< 0.001**

Table SIV.5 – Pairwise comparisons of the flowering patterns of control and ozone-exposed plants within each plant age at the beginning of the fumigation period. The emmeans test analyses how the average number of flowers changes with the exposure to ozone, while the emtrends test takes into account the three-way interaction and compares the slopes of the flowering curves.

Response variable	Pairwise post-hoc (Control - Ozone)		
	Plant age	t-ratio	p-value
		Emmeans	
Number of open flowers	3	-2.64	**0.008**
	4	0.18	0.859
	5	1.07	0.285
	6	1.87	0.062
		Emtrends	
	3	4.53	**< 0.001**
	4	-5.53	**< 0.001**
	5	1.02	0.310
	6	0.03	0.979

Table SIV.6 – The effects of ozone exposure at different plant ages on the number of flower visitors a plant receives in 4-min observation periods. Results from the models that do not consider the offset term for correcting for the number of open flowers. Cells shaded in green and red represent positive and negative effects of ozone, respectively.

Number of visitors in 4-min periods	Not correcting for the number of open flowers						
	Anova (type II)				Pairwise post-hoc (Control - Ozone)		
	Predictor variable	Chisq	df	p-value	Plant age	t-ratio	p-value
All visitors	Treatment	0.03	1	0.868	3	-3.27	**0.001**
	Plant age	5.14	3	0.162	4	-1.64	0.102
	Treatment x Plant age	22.49	3	**<0.001**	5	1.74	0.082
					6	2.45	**0.014**
Bees and syrphids	Treatment	0.01	1	0.909	3	-3.49	**<0.001**
	Plant age	5.25	3	0.154	4	-1.73	0.083
	Treatment x Plant age	25.67	3	**<0.001**	5	2.16	**0.031**
					6	2.39	**0.017**
Only bees	Treatment	0.19	1	0.664	3	-2.96	**0.003**
	Plant age	7.58	3	0.055	4	-2.04	**0.042**
	Treatment x Plant age	27.99	3	**<0.001**	5	2.62	**0.009**
					6	2.88	**0.004**
Honeybees	Treatment	0.09	1	0.766	3	-3.14	**0.002**
	Plant age	11.87	3	**0.008**	4	-1.39	0.166
	Treatment x Plant age	22.39	3	**<0.001**	5	2.65	**0.008**
					6	1.91	0.057
Wild bees	Treatment	0.20	1	0.657	3	-0.91	0.362
	Plant age	2.23	3	0.527	4	-2.22	**0.026**
	Treatment x Plant age	14.65	3	**0.002**	5	1.51	0.132
					6	2.60	**0.009**
Only syrphids	Treatment	0.36	1	0.547	3	-3.12	**0.002**
	Plant age	3.43	3	0.330	4	0.35	0.723
	Treatment x Plant age	10.18	3	**0.017**	5	0.77	0.440
					6	0.26	0.794
Small syrphids	Treatment	0.05	1	0.824	3	-1.66	0.098
	Plant age	3.41	3	0.332	4	1.08	0.282
	Treatment x Plant age	4.65	3	0.199	5	-0.02	0.983
					6	0.89	0.372
Large syrphids	Treatment	0.16	1	0.686	3	-3.42	**<0.001**
	Plant age	1.07	3	0.783	4	0.19	0.846
	Treatment x Plant age	12.93	3	**0.005**	5	1.12	0.265
					6	-0.05	0.958

Table SIV.7 – The effects of ozone exposure at different plant ages on the number of flower visitors a plant receives in 4-min observation periods. Results from the models that correct for the number of open flowers using an offset term. The cell shaded in green represent a positive effect of ozone on the number of large syrphids visiting the flowers.

Number of visitors in 4-min periods	Correcting for the number of open flowers						
	Anova (type II)				Pairwise post-hoc (Control - Ozone)		
	Predictor variable	Chisq	df	p-value	Plant age	t-ratio	p-value
All visitors	Treatment	0.03	1	0.871	3	-0.53	0.594
	Plant age	12.53	3	**0.006**	4	-1.31	0.190
	Treatment x Plant age	4.25	3	0.235	5	0.99	0.321
					6	1.12	0.261
Bees and syrphids	Treatment	0.04	1	0.844	3	-0.90	0.369
	Plant age	11.42	3	**0.010**	4	-1.44	0.150
	Treatment x Plant age	6.40	3	0.094	5	1.52	0.129
					6	1.09	0.274
Only bees	Treatment	0.90	1	0.344	3	-0.31	0.755
	Plant age	9.55	3	**0.023**	4	-1.60	0.111
	Treatment x Plant age	8.82	3	**0.032**	5	1.92	0.055
					6	1.83	0.068
Honeybees	Treatment	0.66	1	0.416	3	-0.96	0.337
	Plant age	7.42	3	0.060	4	-0.94	0.350
	Treatment x Plant age	6.64	3	0.084	5	1.91	0.056
					6	1.34	0.181
Wild bees	Treatment	0.35	1	0.555	3	1.26	0.210
	Plant age	15.48	3	**0.001**	4	-1.72	0.085
	Treatment x Plant age	5.95	3	0.114	5	0.82	0.411
					6	1.06	0.292
Only syrphids	Treatment	1.04	1	0.307	3	-1.57	0.116
	Plant age	7.07	3	0.070	4	0.08	0.935
	Treatment x Plant age	2.45	3	0.484	5	0.12	0.909
					6	-1.00	0.315
Small syrphids	Treatment	0.01	1	0.923	3	-0.53	0.599
	Plant age	8.85	3	**0.031**	4	0.96	0.339
	Treatment x Plant age	2.03	3	0.566	5	-0.87	0.384
					6	0.31	0.761
Large syrphids	Treatment	0.74	1	0.390	3	-2.31	**0.021**
	Plant age	3.64	3	0.303	4	-0.25	0.799
	Treatment x Plant age	6.07	3	0.108	5	0.77	0.444
					6	-0.88	0.380

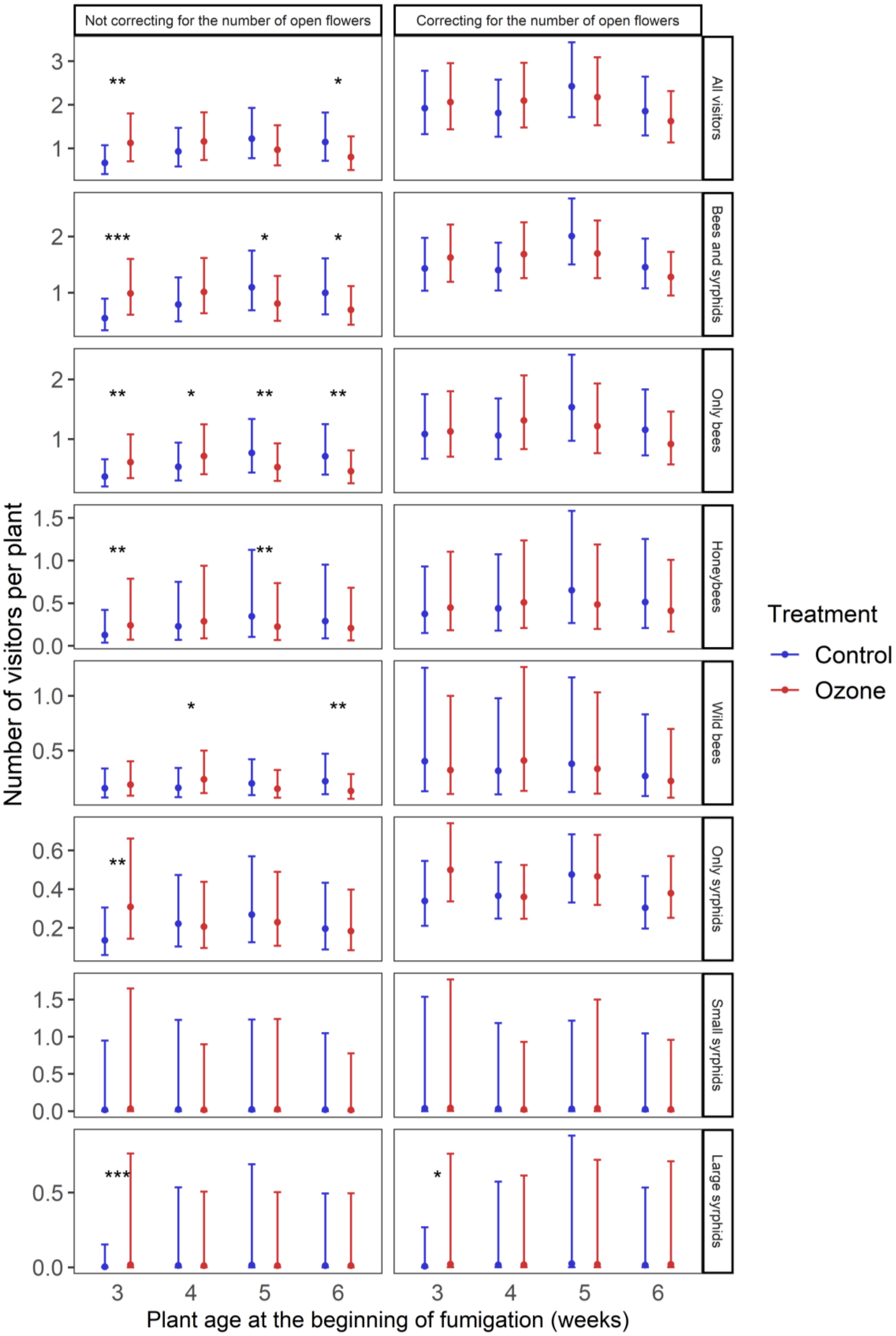


Figure SIV.1 – The effects of ozone exposure at different plant ages on the number of visitors received per plant in 4-min observation periods. The dots are the means and the bars show the 95 % confidence intervals of the fitted models as provided by the emmeans package. Asterisks represent significant differences between treatments within a plant age (* - 0.05 > p ≥ 0.01; ** - 0.01 > p ≥ 0.001; *** - p < 0.001).

Table SIV.8 – The effects of ozone exposure at different plant ages on herbivory. Cells shaded in green and red represent positive and negative effects of ozone, respectively. DAS stands for Days After Sowing.

Response variable	Anova (type II)				Pairwise post-hoc (Control - Ozone)		
	Predictor variable	Chisq	df	p-value	Plant age	t-ratio	p-value
Level of damage by chewing-herbivores	Treatment	2.32	1	0.127	3	-0.61	0.544
	Plant age	2.68	3	0.443	4	-0.17	0.869
	poly(DAS,2)	2.23	2	0.327	5	-0.08	0.937
	Treatment x Plant age	3.98	3	0.263	6	-2.43	**0.015**
Number of aphids	Treatment	0.83	1	0.363	3	0.42	0.678
	Plant age	16.78	3	**<0.001**	4	-2.05	**0.041**
	poly(DAS,2)	1.71	2	0.425	5	1.32	0.187
	Treatment x Plant age	7.20	3	0.066	6	-1.40	0.161
	Plant age x poly(DAS,2)	65.59	6	**<0.001**			
Number of *Lygus pratensis*	Treatment	0.09	1	0.766	3	-1.98	**0.049**
	Plant age	2.99	3	0.393	4	1.20	0.232
	DAS	0.40	1	0.525	5	-1.04	0.299
	Treatment x Plant age	7.16	3	0.067	6	0.97	0.335
Number of aphid midget larva	Treatment	0.17	1	0.683	3	-0.59	0.555
	Plant age	2.88	3	0.410	4	-0.16	0.870
	Number of aphids	22.79	1	**<0.001**	5	-0.80	0.424
	poly(DAS,2)	158.06	2	**<0.001**	6	2.44	**0.015**
	Treatment x Plant age	6.81	3	0.078			

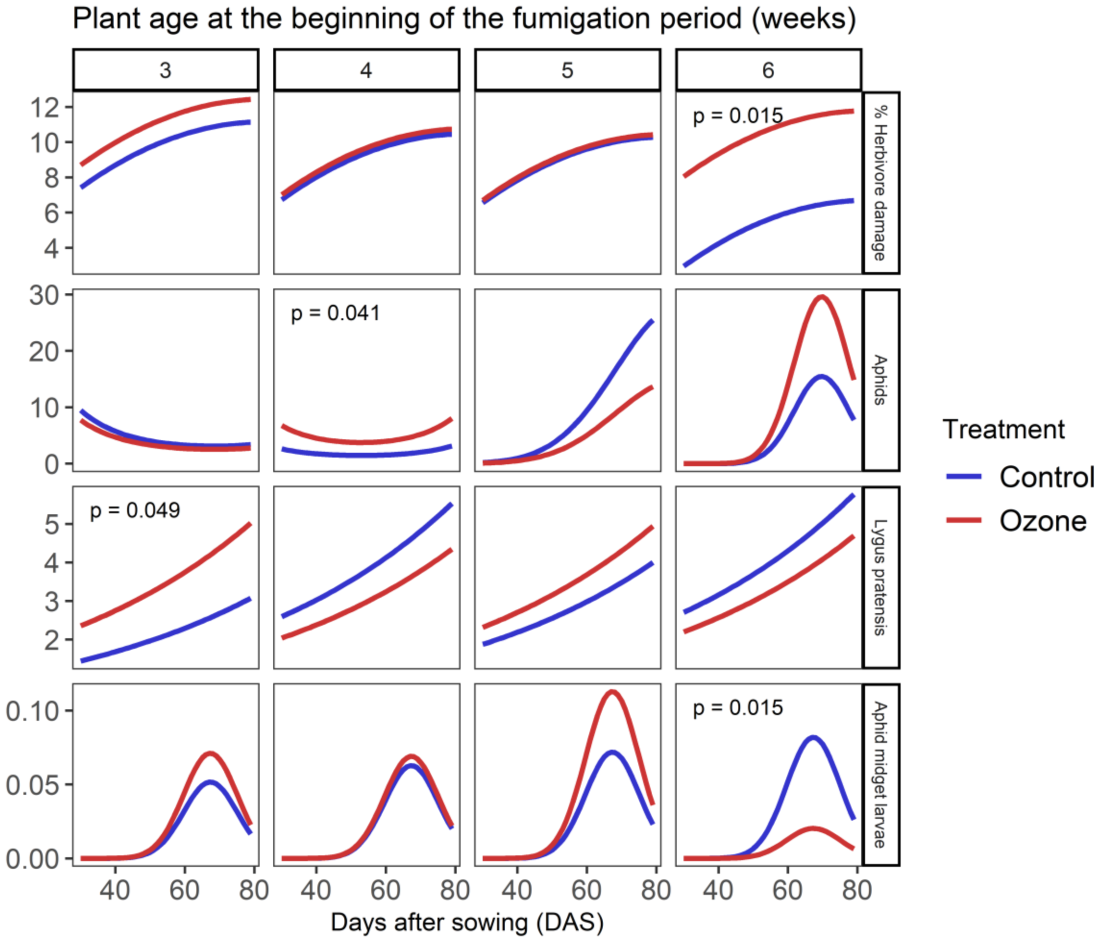
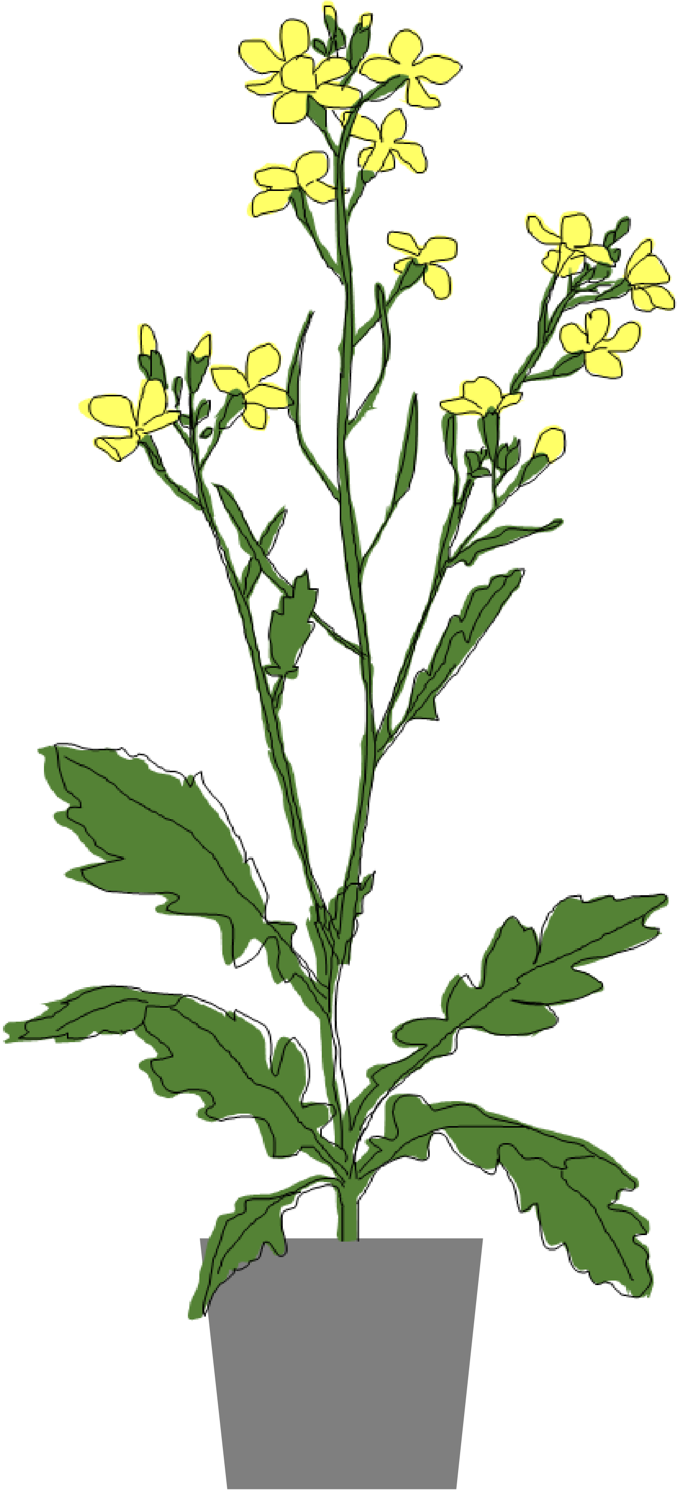


Figure SIV.2 – The effects of ozone exposure at different plant ages on herbivore damage, and the abundance of two herbivores (aphids and *Lygus pratensis* bugs) and a predator (aphid midget larvae). The lines represent the fitted models as provided by the emmeans package. P-values are presented when the effect of ozone in the post-hoc test was ≤ 0.05.

Table SIV.9 – Results of the models predicting the number of seeds produced per plant, including and excluding herbivory, pollination and flowering parameters as predictor variables. Cells shaded in green and red represent positive and negative effects of ozone, respectively.

Model	ΔAICc	Anova (type II)				Pairwise post-hoc (Control - Ozone)		
		Predictor variable	Chisq	df	p-value	Plant age	t-ratio	p-value
Best model	0.00	Treatment	0.17	1	0.681	3	-2.96	**0.004**
		Plant age	0.42	3	0.936	4	-0.05	0.962
		Average number of open flowers	14.67	1	**<0.001**	5	1.21	0.232
		Treatment x Plant age	10.09	3	**0.018**	6	0.75	0.455
Including all predictors tested	5.55	Treatment	0.04	1	0.852	3	-3.03	**0.003**
		Plant age	0.52	3	0.915	4	0.28	0.784
		Average number of aphids	2.56	1	0.109	5	1.15	0.256
		Average herbivore damage	0.21	1	0.650	6	1.02	0.311
		Average visitation rate	0.19	1	0.661			
		Average number of open flowers	12.99	1	**<0.001**			
		Treatment x Plant age	11.02	3	**0.012**			
Including only treatment and plant age as predictors	10.92	Treatment	0.06	1	0.811	3	-3.31	**0.002**
		Plant age	6.46	3	0.091	4	-0.42	0.676
		Treatment x Plant age	18.01	3	**<0.001**	5	1.82	0.073
						6	1.90	0.061



Chapter V

General discussion

The highly oxidative potential of ozone makes tropospheric ozone one of the most detrimental air pollutants. These damaging effects are not only a concern for human health, but also for the health of ecosystems. Using *Sinapis arvensis* as the model plant, I investigated how plant exposure to ozone affects the interactions between plants and insects and ultimately plant reproductive success. I assessed both plant-herbivore (chapters II and IV) and plant-pollinator (chapters III and IV) interactions. My research shows that ozone exposure may confer an advantage to the reproductive success of plants when exposure occurs early in their life cycle and a disadvantage when the plants are exposed at later stages (chapter IV). Flowering phenology was changed by exposing the plants to ozone and the direction of the effects depended on the age of the plant at the time of the exposure (chapters III and IV). The number of flowers revealed a key factor in determining the attractiveness of the plants to flower visitors and, ultimately, the reproductive success of the plants (chapters III and IV). Plants exposed to ozone tended to have higher levels of herbivory (chapter IV). Furthermore, plant exposure to ozone affected the life cycle of the herbivore *Pieris brassicae* and the effects depended on air temperature and the duration of the exposure (chapter II).

Plant exposure to ozone and consequences for plant reproduction

The success of an annual plant is measured by the number and fitness of its offspring. Plant stress reduces the chances of success, while plant tolerance to stress improves the chances of success. Reallocation of resources to reproduction and alteration of the time of flowering are strategies that plants can use under adverse conditions in order to maximize reproductive success (Kazan and Lyons, 2016). According to my research, ozone stress can be included in the plethora of stresses that induce flowering. Plant exposure to ozone accelerated flowering of plants that were lacking other flowering-inducing stimuli, such as light and temperature (chapter III) and of plants that were exposed at an early age (chapter IV). Exposing plants closer to the natural time of onset of flowering did not result in an acceleration of flowering (chapter IV). Ozone exposure also resulted in an increased amount of open flowers on plants exposed early in their lives and a tendency for a decreased amount of flowers as plants were exposed at progressively later ages (chapter IV).

For entomophilous plants, an issue that may arise by accelerating flowering is the creation of mismatches between the plants and their insect pollinators. In other words, acceleration of flowering will only constitute an advantage to the plants when this strategy allows for keeping synchronicity with their pollinators. For *Sinapis arvensis*, a temporal mismatch with its pollinators is not a real issue, since this plant is a pollinator generalist that blooms for a relatively long period in the middle of the pollinating season. However, a temporal mismatch through accelerated flowering is a risk for plants that have short flowering periods and that bloom early in the pollinating season, especially if they are pollinator specialists. In the face of climate change, insect phenology is expected to accelerate more than plant phenology (Thackeray et al., 2016). In this case, plant exposure to ozone may confer an advantage to plants, by increasing the extent of the phenological shift and restoring synchronicity.

In my research, the increased investment in flowering came at the expenses of decreased investment in vegetative mass (chapter III). Besides, plants exposed to ozone had chlorotic/necrotic spots in their leaves (chapter II). So, was the increased investment in reproduction enough to counterbalance the damaging effects of ozone?

According to my studies, *Sinapis arvensis* plants may even overcompensate. In plants that were grown in the field after fumigation, exposure to ozone resulted in higher reproductive success of plants that were exposed early in their life (3 weeks after sowing), while a gradual tendency for the opposite effect was observed as plants were exposed at later ages (chapter IV). On the other hand, in plants that were kept in the greenhouse after fumigation and that were exposed to ozone when they were 4 weeks old, only one of the four groups of plants showed decreased reproductive success, while the others showed no effect (chapter III). In all cases, the number of flowers in the plant were important factors influencing success. Another factor that may have contributed to the observed differences, and particularly to the negative effects of ozone, is the air relative humidity. As plants grow, relative humidity in the plant canopy is expected to increase (Hardwick et al., 2015), and so it happened in my research (chapter IV). High relative humidity increases ozone uptake by the plants, which in turn correlates well with negative effects of ozone (Emberson et al., 2018; Gong et al., 2021).

Reports of negative effects of ozone exposure on reproductive success are common, while positive effects have rarely been reported (Leisner and Ainsworth, 2012). So, what is so special about *Sinapis arvensis*?

Sinapis arvensis is an indeterminate plant. Indeterminate plants prolong flowering when the reproductive success is low and, therefore, have higher chances of compensating for stress (Black et al., 2000). Also, plant age is key, and most studies have not used plants as young as the ones used in my experiments. However, it might be the case that the exposure to ozone, by accelerating flowering, made the reproductive stage overlap with the best available weather conditions for fertilization and fruit development, therefore increasing success by chance.

An alternative reason for the differences in success observed in exposed and non-exposed plants would be the changes in plant-insect interactions resulting from plant exposure to ozone. However, that does not seem to be the case in my studies.

Plant exposure to ozone and herbivores

Plant exposure to ozone changed several parameters in the life cycle of *Pieris brassicae*, a Brassicaceae herbivore specialist. *Pieris brassicae* butterflies preferred plants that did not undergo ozone stress as oviposition sites. However, when eggs were deposited on ozone-exposed plants they were more likely to get to caterpillar stage. On the hand, they took longer to hatch and the growth of the caterpillars was reduced.

The decrease in growth showed by the caterpillars may be a result of poorer nutritional quality of the plants following exposure, as demonstrated for other plants (Couture and Lindroth, 2012; Couture et al., 2012; Khaling et al., 2015; Peltonen et al., 2010). In fact, the reduced nutritional quality may be the reason for increased consumption shown in other studies (Abu ElEla et al., 2018; Jones and Coleman, 1988). Increased consumption as a response to reduced quality may be at the base of the increased plant damage by chewing herbivores that I observed in the field in plants exposed to ozone (chapter IV).

While the preference of *Pieris brassicae* butterflies was rather clear, the effect of plant exposure to ozone on the number of sucking herbivores was weaker, but when differences existed, they showed that aphids and *Lygus pratensis* bugs do rather well in ozone-exposed plants (chapter IV).

Plant exposure to ozone and pollination

Success of entomophilous plants is dependent on the services provided by insect pollinators. Therefore, disrupted interactions between the plant and its pollinators would strongly affect reproduction. In my research, whenever the number of flower visits received by the plants was affected by exposure to ozone, this was due to changes in the number of open flowers (chapters III and IV). More flowers constituted a stronger attraction signal, while less flowers constituted a weaker attraction signal. An exception to this pattern occurred for the number of flower visits performed by large syrphid flies to young exposed plants, which was higher on ozone-exposed plants than on the control plants regardless of flower numbers (chapter IV). Also, honeybees spent more time in ozone-exposed plants than on control plants (chapter III). This preference for ozone-exposed plants may be related to an increase in the amount and concentration of the nectar produced, as observed for broad bean plants (Stabler, 2016).

A recent study showed that, unlike what happens to other plant species, exposure to ozone does not have a strong effect on the emission of volatile organic compounds (VOCs) of *Sinapis arvensis* (Saunier and Blande, 2019). A lack of a strong effect on the emission of these VOCs would justify the absence of a stronger response of the pollinators to ozone-exposed *Sinapis arvensis* plants. A different outcome might be expected when ozone is present during the plant-pollinator interaction, since the emitted VOCs could react with ozone in the atmosphere, changing the chemical cues used by the pollinators, possibly disrupting the communication. Also, the contact of the pollinators with ozone might affect their olfactory detection and perception (Démares et al., 2022; Vanderplanck et al., 2021), therefore changing their response to the chemical cues emitted by the plants.

Factors influencing the effects of ozone

Duration of exposure

The dose of plant exposure to ozone may vary either by changing the concentration of ozone or by changing the duration of the exposure. Previous studies that addressed the plant-mediated effects of ozone on plant-insect interactions have only considered the former (Fuentes et al., 2013; Jackson et al., 1999; Khaling et al., 2016; Khaling et al., 2015). In chapter II, I showed that the duration of the exposure is an important factor when considering the effects of ozone. While exposing plants to 120 ppb of ozone for 5 days, 6 hours per day, had effects on all parameters of the life cycle of *Pieris brassicae* studied, only the egg stage (survival and duration of the stage) were affected when the plants were exposed for only 1 day.

Timing of exposure

It was suggested (Agathokleous et al., 2019b) and demonstrated for some plant species (Agathokleous et al., 2019a) that plant response to ozone exposure follows an hormetic model. This means that exposing plants to low doses of ozone might have a stimulating effect, while increasing the dose of exposure beyond a species-specific threshold, the effects would then be negative. Considering the results shown in chapter IV, it can be argued that the direction of the effects may not only depend on the dose of ozone exposure, but also on the timing of the exposure. Keeping the dose of exposure to ozone constant (120 ppb, 6 hours/day, 7 consecutive days), but exposing plants at different points of their development, a clear pattern emerged: the effects of the exposure were positive for young plants and gradually tended to be negative for plants exposed at later ages. This was true for both the change in the number of flowers and the change in reproductive performance. This means that, at least for *Sinapis arvensis*, the threshold for adverse effects may be dependent on the age of the plant.

Air temperature

In chapter II, I showed that the effects of ozone were exacerbated when the temperature was high. It is not clear whether this happens because high temperatures following ozone exposure intensify the effects of ozone stress in the plant, possibly changing its nutritional value, or because high temperatures increase the growth rate of the caterpillars, therefore speeding up the divergence of caterpillars fed plants with different nutritional values. Independently of the reason, this is bad news, considering the current increase in global average temperature.

Also, experiencing high temperatures before ozone exposure increases the susceptibility of plants to ozone (Menser et al., 1963). Surprisingly, very few studies have addressed the interactive effects of ozone and air temperature during exposure, but they show that, when interactive effects occur, they are antagonistic (Kasurinen et al., 2012; Maenpaa et al., 2011).

Research limitations

In the recent past, many studies on the effects of ozone on plants and plant-insect interactions have used (compressed) air to generate ozone by electric discharge (Cui et al., 2014; Guo et al., 2018; Li et al., 2016; Mofikoya et al., 2018; Telesnicki et al., 2018). Therefore, only towards the end of my research work, I became aware that generating ozone by electric discharge using compressed air as the feed gas produces oxides of nitrogen. This results in confounding effects on ozone-exposed plants due to absorption of nitrogen. The production of most nitrogen oxides seems to be negligible though. Of greater importance is the production of higher oxides of nitrogen, namely N_2O_5. This compound seems to increase considerably, but keeping two orders of magnitude below the ozone concentration (Kogelschatz and Eliasson, 2018). Whether this amount of N_2O_5 is negligible or not is unknown, since there is very little evidence of the effects of this oxide of nitrogen on plants.

In fact, in a study from Mortensen and Jorgensen (1996), the damaged leaf dry weight of one of the spring wheat cultivars studied was higher in plants fumigated with ozone produced by UV-lamps (that do not produce any NOx) than in plants fumigated with ozone produced by an electric discharge generator fed dry air. Therefore, there is no striking evidence that the amount of NOx produced by electric discharge ozone generators running on air would have negative effects on the plants.

However, to avoid creating confounding effects, two other methods for enhancing the ozone concentration in the air would be preferable to the one used in my research: 1) continue using compressed air as the feed gas for the ozone generator but bubbling the resulting air through water to get rid of the nitrogen oxides or 2) substitute the compressed air for pure oxygen, this way avoiding the production of nitrogen oxides.

Future directions

Far from answering all the questions, research tends to raise additional questions. In this section, I aim at specifying some areas of research on the effects of ozone exposure on plants and plant-insect interactions that I consider need (further) investment:

- As mentioned before, acceleration of flowering by exposure to ozone may put the pollination of plants at risk, by establishing mismatches with their pollinators. Pollinator specialist plants and plants that bloom early in the pollinating season are naturally at greater risk. The anticipation of the peak of exposure to ozone from Summer to Spring (Neufeld et al., 2019) further increases the threat to early bloomers. Future studies on the effects of ozone on plant phenology should target these plants and assess whether synchronicity with pollinators is disrupted by exposure to ozone.

- Despite the considerable amount of studies on the effects of tropospheric ozone on plant reproduction and crop yield, little is known on the fitness of the offspring of ozone-exposed plants. The few available studies report both advantages and disadvantages of plant exposure to ozone on the germination of their seeds, but in most cases plant exposure to ozone occurred also during or after seed formation, making it impossible to distinguish between maternal effects and direct effects on the seeds (Black et al., 2000; Bosac et al., 1998; Darbah et al., 2008; Landesmann et al., 2013). Also important, but rarely explored, is the vigour of the seedlings resulting from those seeds (Bosac et al., 1998). Changes in the fitness of the offspring are one way ozone may shift plant species dominance and alter the structure of plant communities (Grulke and Heath, 2020; Martinez-Ghersa et al., 2017).

- The oxidative properties of ozone make it a very good anti-microbial agent. Therefore, ozone treatment has been studied to be used to the advantage of the agro and food industries, as a method for enhancing seed germination (Pandiselvam et al., 2020) or extending shelf-life (Afonso et al., 2022; Meireles et al., 2016). In my studies, ozone exposure had opposite consequences for plant reproduction depending on the plant age at the time of exposure, with advantages for plants exposed at early ages. Studies with other plant species exposed at different ages are necessary to see if the same pattern observed here applies. If reproduction is also boosted in crop plants exposed to ozone at early ages, this could open new doors in the field of horticulture.

- My research on the plant-mediated effects of ozone on the life of the herbivore *Pieris brassicae* shows that the growth of caterpillars feeding on ozone-exposed plants is reduced. However, the results do not show whether this would translate in a longer caterpillar stage or in smaller pupae or both. Following this last stage of development of the herbivore, as well as the fate of the next generation would add important information to have a full picture on the plant-mediated effects of ozone in the life of an herbivore.

- By altering the dominance of plants in a given community, ozone exposure might also be affecting the structure of food webs (Martinez-Ghersa et al., 2017). However, studies on the direct impact of ozone on insects is largely missing. Is the diversity and dominance of the herbivores changed by direct exposure of the herbivores to ozone? Is insect attraction to plants impaired by ozone?

Conclusions

While there is no question that ozone can be highly detrimental to plants and to their interactions with insects, I showed that this is not always the case. My research shows that effects of ozone depend on the duration and timing of the exposure and on temperature. The most detrimental effects were observed for longer exposures and exposures occurring close or post beginning of flowering. Higher temperature following exposure enhanced the negative effects of ozone. On the other hand, exposing the plants earlier in their life cycle revealed advantageous to the plants: they shifted their investment to reproduction, ending up with a higher reproductive performance through overcompensation.

Also, the acceleration of flowering that occurred following exposure of young plants to ozone may reveal being beneficial or detrimental to the plants depending on their interactions with insect pollinators and how their pollinators will change their phenology in response to climate change.

In my studies, plant reproduction was not strongly affected by the changes in the interactions with the insects. On the other hand, the performance of *Pieris brassicae* was strongly affected by the changes produced by ozone in the plants.

Plant sensitivity to ozone is species-specific. By selectively affecting plants, ozone may increase the competitiveness of some over others, changing the structure of plant communities. By accelerating flowering, as shown in my research, ozone may cause mismatches between plant and pollinator activities. By reducing the performance of herbivores, ozone may lead to changes in the organization of foods webs. Overall, this suggests that ecosystem disturbance by high levels of tropospheric ozone may be a threaten to its stability.

References

Abu ElEla, S.A., Agathokleous, E., Koike, T., 2018. Growth and nutrition of *Agelastica coerulea* (Coleoptera: Chrysomelidae) larvae changed when fed with leaves obtained from an O_3-enriched atmosphere. Environmental Science and Pollution Research 25, 13186-13194.

Afonso, R.B., Moreira, R.H.R., de Almeida, P.L.R., 2022. Can ozone be used as antimicrobial in the dairy industry? A systematic review. Journal of Dairy Science 105, 1051-1057.

Agathokleous, E., Araminiene, V., Belz, R.G., Calatayud, V., De Marco, A., Domingos, M., Feng, Z.Z., Hoshika, Y., Kitao, M., Koike, T., Paoletti, E., Saitanis, C.J., Sicard, P., Calabrese, E.J., 2019a. A quantitative assessment of hormetic responses of plants to ozone. Environmental Research 176, 10.

Agathokleous, E., Belz, R.G., Calatayud, V., De Marco, A., Hoshika, Y., Kitao, M., Saitanis, C.J., Sicard, P., Paoletti, E., Calabrese, E.J., 2019b. Predicting the effect of ozone on vegetation via linear non-threshold (LNT), threshold and hormetic dose-response models. Science of The Total Environment 649, 61-74.

Agathokleous, E., Feng, Z.Z., Oksanen, E., Sicard, P., Wang, Q., Saitanis, C.J., Araminiene, V., Blande, J.D., Hayes, F., Calatayud, V., Domingos, M., Veresoglou, S.D., Penuelas, J., Wardle, D.A., De Marco, A., Li, Z.Z., Harmens, H., Yuan, X.Y., Vitale, M., Paoletti, E., 2020. Ozone affects plant, insect, and soil microbial communities: A threat to terrestrial ecosystems and biodiversity. Science Advances 6, 17.

Agathokleous, E., Sakikawa, T., Abu Elela, S.A., Mochizuki, T., Nakamura, M., Watanabe, M., Kawamura, K., Koike, T., 2017. Ozone alters the feeding behavior of the leaf beetle Agelastica coerulea (Coleoptera: Chrysomelidae) into leaves of Japanese white birch (Betula platyphylla var. japonica). Environmental Science and Pollution Research 24, 17577-17583.

Amundson, R.G., Raba, R.M., Schoettle, A.W., Reich, P.B., 1986. Response of soybean to low concentrations of ozone. 2. Effects on growth, biomass allocation, and flowering. Journal of Environmental Quality 15, 161-167.

Ashmore, M.R., 2005. Assessing the future global impacts of ozone on vegetation. Plant Cell and Environment 28, 949-964.

Avnery, S., Mauzerall, D.L., Liu, J.F., Horowitz, L.W., 2011a. Global crop yield reductions due to surface ozone exposure: 1. Year 2000 crop production losses and economic damage. Atmospheric Environment 45, 2284-2296.

Avnery, S., Mauzerall, D.L., Liu, J.F., Horowitz, L.W., 2011b. Global crop yield reductions due to surface ozone exposure: 2. Year 2030 potential crop production losses and economic damage under two scenarios of O_3 pollution. Atmospheric Environment 45, 2297-2309.

Barragan-Fonseca, K.Y., Van Loon, J.J.A., Dicke, M., Lucas-Barbosa, D., 2020. Use of visual and olfactory cues of flowers of two brassicaceous species by insect pollinators. Ecological Entomology 45, 45-55.

Barton, K., 2018. MuMIn: Multi-Model Inference. R package version 1.42.1.

Bates, D., Maechler, M., Bolker, B., Walker, S., 2015. Fitting linear mixed-effects models using lme4. Journal of Statistical Software 67, 1-48.

Black, V.J., Black, C.R., Roberts, J.A., Stewart, C.A., 2000. Impact of ozone on the reproductive development of plants. New Phytologist 147, 421-447.

Bolsinger, M., Lier, M.E., Hughes, P.R., 1992. Influence of ozone air-pollution on plant-herbivore interactions. Part 2: Effects of ozone on feeding preference, growth and consumption rates of monarch butterflies (*Danaus plexippus*). Environmental Pollution 77, 31-37.

Bommarco, R., Marini, L., Vaissiere, B.E., 2012. Insect pollination enhances seed yield, quality, and market value in oilseed rape. Oecologia 169, 1025-1032.

Bosac, C., Black, V.J., Roberts, J.A., Black, C.R., 1998. Impact of ozone on seed yield and quality and seedling vigour in oilseed rape (Brassica napus L.). Journal of Plant Physiology 153, 127-134.

Brooks, M.E., Kristensen, K., van Benthem, K.J., Magnusson, A., Berg, C.W., Nielsen, A., Skaug, H.J., Maechler, M., Bolker, B.M., 2017. glmmTMB Balances speed and flexibility among packages for zero-inflated generalized linear mixed modeling. The R Journal 9, 378-400.

Brown, K.A., Roberts, T.M., 1988. Effects of ozone on foliar leaching in norway spruce (*Picea abies* L. Karst) - confounding factors due to NOx production during ozone generation. Environmental Pollution 55, 55-73.

Bruce, T.J.A., Pickett, J.A., 2011. Perception of plant volatile blends by herbivorous insects - Finding the right mix. Phytochemistry 72, 1605-1611.

Bruinsma, M., Lucas-Barbosa, D., ten Broeke, C.J.M., van Dam, N.M., van Beek, T.A., Dicke, M., van Loon, J.J.A., 2014. Folivory affects composition of nectar, floral odor and modifies pollinator behavior. Journal of Chemical Ecology 40, 39-49.

Burkle, L.A., Marlin, J.C., Knight, T.M., 2013. Plant-pollinator interactions over 120 years: loss of species, co-occurrence, and function. Science 339, 1611-1615.

Burkle, L.A., Runyon, J.B., 2017. The smell of environmental change: using floral scent to explain shifts in pollinator attraction. Applications in Plant Sciences 5, 10.

Byers, D.L., 2017. Studying plant-pollinator interactions in a changing climate: A review of approaches. Applications in Plant Sciences 5, 15.

Changey, F., Bagard, M., Souleymane, M., Lerch, T.Z., 2018. Cascading effects of elevated ozone on wheat rhizosphere microbial communities depend on temperature and cultivar sensitivity. Environmental Pollution 242, 113-125.

Chittka, L., Raine, N.E., 2006. Recognition of flowers by pollinators. Current Opinion in Plant Biology 9, 428-435.

Cook, B., Haverkamp, A., Hansson, B.S., Roulston, T.a., Lerdau, M., Knaden, M., 2020a. Pollination in the anthropocene: A moth can learn ozone-altered floral blends. Journal of Chemical Ecology 46, 987-996.

Cook, D.F., Voss, S.C., Finch, J.T.D., Rader, R.C., Cook, J.M., Spurr, C.J., 2020b. The role of flies as pollinators of horticultural crops: an australian case study with worldwide relevance. Insects 11, 31.

Couture, J.J., Lindroth, R.L., 2012. Atmospheric change alters performance of an invasive forest insect. Global Change Biology 18, 3543-3557.

Couture, J.J., Meehan, T.D., Lindroth, R.L., 2012. Atmospheric change alters foliar quality of host trees and performance of two outbreak insect species. Oecologia 168, 863-876.

Crawley, M.J., 1989. Insect herbivores and plant population dynamics. Annual review of entomology 34, 531-562.

Cui, H.Y., Su, J.W., Wei, J.N., Hu, Y.J., Ge, F., 2014. Elevated O_3 enhances the attraction of whitefly-infested tomato plants to *Encarsia formosa*. Scientific Reports 4, 6.

Cyr, H., Face, M.L., 1993. Magnitude and patterns of herbivory in aquatic and terrestrial ecosystems. Nature 361, 148-150.

Darbah, J.N.T., Kubiske, M.E., Nelson, N., Oksanen, E., Vapaavuori, E., Karnosky, D.F., 2008. Effects of decadal exposure to interacting elevated CO_2 and/or O_3 on paper birch (*Betula papyrifera*) reproduction. Environmental Pollution 155, 446-452.

David, W.A.L., Gardiner, B.O.C., 2009. Oviposition and the hatching of the eggs of Pieris brassicae (L.) in a laboratory culture. Bulletin of Entomological Research 53, 91-109.

Démares, F., Gibert, L., Creusot, P., Lapeyre, B., Proffit, M., 2022. Acute ozone exposure impairs detection of floral odor, learning, and memory of honey bees, through olfactory generalization. Science of The Total Environment 827, 154342.

Descamps, C., Maree, S., Hugon, S., Quinet, M., Jacquemart, A.L., 2020. Species-specific responses to combined water stress and increasing temperatures in two bee-pollinated congeners (Echium, Boraginaceae). Ecology and Evolution, 13.

Dicke, M., van Loon, J.J.A., Soler, R., 2009. Chemical complexity of volatiles from plants induced by multiple attack. Nature Chemical Biology 5, 317-324.

Dolferus, R., Ji, X.M., Richards, R.A., 2011. Abiotic stress and control of grain number in cereals. Plant Science 181, 331-341.

Dotterl, S., Vater, M., Rupp, T., Held, A., 2016. Ozone differentially affects perception of plant volatiles in western honey bees. Journal of Chemical Ecology 42, 486-489.

Drogoudi, P.D., Ashmore, M.R., 2000. Does elevated ozone have differing effects in flowering and deblossomed strawberry? New Phytologist 147, 561-569.

Duque, L., Poelman, E.H., Steffan-Dewenter, I., 2019. Plant-mediated effects of ozone on herbivores depend on exposure duration and temperature. Scientific Reports 9, 19891.

Duque, L., Poelman, E.H., Steffan-Dewenter, I., 2021. Effects of ozone stress on flowering phenology, plant-pollinator interactions and plant reproductive success. Environmental Pollution 272, 115953.

EEA, 2006. Air pollution by ozone in Europe in summer 2005, EEA Technical Report. European Environment Agency, Copenhagen, Denmark.

EEA, 2010. Air pollution by ozone across Europe during summer 2009, EEA Technical Report. European Environment Agency, Copenhagen, Denmark.

EEA, 2014. Air pollution by ozone across Europe during summer 2013, EEA Technical Report. European Environment Agency, Copenhagen, Denmark.

Emberson, L.D., Pleijel, H., Ainsworth, E.A., van den Berg, M., Ren, W., Osborne, S., Mills, G., Pandey, D., Dentener, F., Buker, P., Ewert, F., Koeble, R., Van Dingenen, R., 2018. Ozone effects on crops and consideration in crop models. European Journal of Agronomy 100, 19-34.

EPA, 1990. Clean Air Act, Amendments of 1990.

EU, 2008. Directive 2008/50/EC of the European Parliament and of the Council of 21 May 2008 on ambient air quality and cleaner air for Europe. Official Journal of The European Union.

Farre-Armengol, G., Filella, I., Llusia, J., Penuelas, J., 2015. Relationships among floral VOC emissions, floral rewards and visits of pollinators in five plant species of a Mediterranean shrubland. Plant Ecology and Evolution 148, 90-99.

Farre-Armengol, G., Penuelas, J., Li, T., Yli-Pirila, P., Filella, I., Llusia, J., Blande, J.D., 2016. Ozone degrades floral scent and reduces pollinator attraction to flowers. New Phytologist 209, 152-160.

Fatouros, N.E., Cusumano, A., Danchin, E.G.J., Colazza, S., 2016. Prospects of herbivore egg-killing plant defenses for sustainable crop protection. Ecology and Evolution 6, 6906-6918.

Fiscus, E.L., Booker, F.L., Burkey, K.O., 2005. Crop responses to ozone: uptake, modes of action, carbon assimilation and partitioning. Plant, Cell & Environment 28, 997-1011.

Fitzky, A.C., Sandén, H., Karl, T., Fares, S., Calfapietra, C., Grote, R., Saunier, A., Rewald, B., 2019. The interplay between ozone and urban vegetation - BVOC emissions, ozone deposition, and tree ecophysiology. Frontiers in Forests and Global Change 2.

Fogg, G.E., 1950. *Sinapis arvensis* L. Journal of Ecology 38, 415-429.

Forrest, J.R.K., Thomson, J.D., 2011. An examination of synchrony between insect emergence and flowering in Rocky Mountain meadows. Ecological Monographs 81, 469-491.

Fox, J., Weisberg, S., 2019. An {R} Companion to Applied Regression, Third ed. Sage, Thousand Oaks, CA, USA.

Freiwald, V., Haikio, E., Julkunen-Tiitto, R., Holopainen, J.K., Oksanen, E., 2008. Elevated ozone modifies the feeding behaviour of the common leaf weevil on hybrid aspen through shifts in developmental, chemical, and structural properties of leaves. Entomologia Experimentalis Et Applicata 128, 66-72.

Fuentes, J.D., Roulston, T.H., Zenker, J., 2013. Ozone impedes the ability of a herbivore to find its host. Environmental Research Letters 8, 5.

Fuhrer, J., Martin, M.V., Mills, G., Heald, C.L., Harmens, H., Hayes, F., Sharps, K., Bender, J., Ashmore, M.R., 2016. Current and future ozone risks to global terrestrial biodiversity and ecosystem processes. Ecology and Evolution 6, 8785-8799.

Gallai, N., Salles, J.M., Settele, J., Vaissiere, B.E., 2009. Economic valuation of the vulnerability of world agriculture confronted with pollinator decline. Ecological Economics 68, 810-821.

Gillespie, C., Stabler, D., Tallentire, E., Goumenaki, E., Barnes, J., 2015. Exposure to environmentally-relevant levels of ozone negatively influence pollen and fruit development. Environmental Pollution 206, 494-501.

Girling, R.D., Lusebrink, I., Farthing, E., Newman, T.A., Poppy, G.M., 2013. Diesel exhaust rapidly degrades floral odours used by honeybees. Scientific Reports 3, 5.

Giron-Calva, P.S., Li, T., Blande, J.D., 2016. Plant-plant interactions affect the susceptibility of plants to oviposition by pests but are disrupted by ozone pollution. Agriculture Ecosystems & Environment 233, 352-360.

Giron-Calva, P.S., Li, T., Blande, J.D., 2017. Volatile-mediated interactions between cabbage plants in the field and the impact of ozone pollution. Journal of Chemical Ecology 43, 339-350.

Gong, C., Yue, X., Liao, H., Ma, Y.M., 2021. A humidity-based exposure index representing ozone damage effects on vegetation. Environmental Research Letters 16, 10.

Grass, I., Bohle, V., Tscharntke, T., Westphal, C., 2018. How plant reproductive success is determined by the interplay of antagonists and mutualists. Ecosphere 9, 15.

Griese, E., Dicke, M., Hilker, M., Fatouros, N.E., 2017. Plant response to butterfly eggs: inducibility, severity and success of egg-killing leaf necrosis depends on plant genotype and egg clustering. Scientific Reports 7, 12.

Grulke, N.E., Heath, R.L., 2020. Ozone effects on plants in natural ecosystems. Plant Biology 22, 12-37.

Guo, H.G., Sun, Y.C., Yan, H.Y., Li, C.Y., Ge, F., 2018. O_3-Induced Leaf Senescence in Tomato Plants Is Ethylene Signaling-Dependent and Enhances the Population Abundance of Bemisia tabaci. Frontiers in Plant Science 9, 14.

Hardwick, S.R., Toumi, R., Pfeifer, M., Turner, E.C., Nilus, R., Ewers, R.M., 2015. The relationship between leaf area index and microclimate in tropical forest and oil palm plantation: Forest disturbance drives changes in microclimate. Agricultural and Forest Meteorology 201, 187-195.

Hartig, F., 2018. DHARMa: Residual diagnostics for hierarchical (multi-level / mixed) regression models. R package version 0.2.0.

Hartig, F., 2020. DHARMa: Residual diagnostics for hierarchical (multi-level / mixed) regression models. R package version 0.3.1.

Hayes, F., Jones, M.L.M., Mills, G., Ashmore, M., 2007. Meta-analysis of the relative sensitivity of semi-natural vegetation species to ozone. Environmental Pollution 146, 754-762.

Hayes, F., Williamson, J., Mills, G., 2012. Ozone pollution affects flower numbers and timing in a simulated BAP priority calcareous grassland community. Environmental Pollution 163, 40-47.

Hilker, M., Meiners, T., 2011. Plants and insect eggs: How do they affect each other? Phytochemistry 72, 1612-1623.

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, 174-186.

Holopainen, J.K., 2002. Aphid response to elevated ozone and CO_2. Entomologia Experimentalis Et Applicata 104, 137-142.

Holopainen, J.K., Kössi, S., 1998. Variable growth and reproduction response of the spruce shoot aphid, Cinara pilicornis, to increasing ozone concentrations. Entomologia Experimentalis Et Applicata 87, 109-113.

Holton, M.K., Lindroth, R.L., Nordheim, E.V., 2003. Foliar quality influences tree-herbivore-parasitoid interactions: effects of elevated CO_2, O_3, and plant genotype. Oecologia 137, 233-244.

IPBES, 2016. The assessment report of the Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services on pollinators, pollination and food production in: Potts, S.G., Imperatriz-Fonseca, V.L., Ngo, H.T. (Eds.). Secretariat of the Intergovernmental Science-Policy Platform on Biodversity and Ecosystem Services, Bonn, Germany, p. 552.

Jackson, D.M., Heagle, A.S., Eckel, R.V.W., 1999. Ovipositional response of tobacco hornworm moths (Lepidoptera : Sphingidae) to tobacco plants grown under elevated levels of ozone. Environmental Entomology 28, 566-571.

Jackson, D.M., Rufty, T.W., Heagle, A.S., Severson, R.F., Eckel, R.V.W., 2000. Survival and development of tobacco hornworm larvae on tobacco plants grown under elevated levels of ozone. Journal of Chemical Ecology 26, 1-19.

Jaenike, J., 1978. Optimal oviposition behavior in phytophagous insects. Theoretical Population Biology 14, 350-356.

Jaworski, T., Hilszczański, J., 2013. The effect of temperature and humidity changes on insects development and their impact on forest ecosystems in the context of expected climate change. Forest Research Papers 74, 345-355.

Jondrup, P.M., Barnes, J.D., Port, G.R., 2002. The effect of ozone fumigation and different *Brassica rapa* lines on the feeding behaviour of *Pieris brassicae* larvae. Entomologia Experimentalis Et Applicata 104, 143-151.

Jones, C.G., Coleman, J.S., 1988. Plant stress and insect behavior: cottonwood, ozone and the feeding and oviposition preference of a beetle. Oecologia 76, 51-56.

Kasurinen, A., Biasi, C., Holopainen, T., Rousi, M., Maenpaa, M., Oksanen, E., 2012. Interactive effects of elevated ozone and temperature on carbon allocation of silver birch (*Betula pendula*) genotypes in an open-air field exposure. Tree Physiology 32, 737-751.

Kazan, K., Lyons, R., 2016. The link between flowering time and stress tolerance. Journal of Experimental Botany 67, 47-60.

Kearns, C.A., Inouye, D.W., Waser, N.M., 1998. Endangered mutualisms: the conservation of plant-pollinator interactions. Annual Review of Ecology and Systematics 29, 83-112.

Khaling, E., Li, T., Holopainen, J.K., Blande, J.D., 2016. Elevated ozone modulates herbivore-induced volatile emissions of brassica nigra and alters a tritrophic interaction. Journal of Chemical Ecology 42, 368-381.

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.

Klein, A.M., Vaissiere, B.E., Cane, J.H., Steffan-Dewenter, I., Cunningham, S.A., Kremen, C., Tscharntke, T., 2007. Importance of pollinators in changing landscapes for world crops. Proceedings of the Royal Society B-Biological Sciences 274, 303-313.

Kogelschatz, U., Eliasson, B., 2018. Ozone Generation and Applications, in: Chang, J.-S., Kelly Arnold, J., Crowley Joseph, M. (Eds.), Handbook of electrostatic processes. CRC Press, Boca Raton, FL, USA, pp. 581-606.

Kopper, B.J., Lindroth, R.L., 2003. Responses of trembling aspen (*Populus tremuloides*) phytochemistry and aspen blotch leafminer (*Phyllonorycter tremuloidiella*) performance to elevated levels of atmospheric CO_2 and O_3. Agricultural and Forest Entomology 5, 17-26.

Kopper, B.J., Lindroth, R.L., Nordheim, E.V., 2001. CO_2 and O_3 effects on paper birch (Betulaceae : *Betula papyrifera*) phytochemistry and whitemarked tussock moth (Lymantriidae : *Orgyia leucostigma*) performance. Environmental Entomology 30, 1119-1126.

Kudo, G., Ida, T.Y., 2013. Early onset of spring increases the phenological mismatch between plants and pollinators. Ecology 94, 2311-2320.

Landesmann, J.B., Gundel, P.E., Martínez-Ghersa, M.A., Ghersa, C.M., 2013. Ozone exposure of a weed community produces adaptive changes in seed populations of *Spergula arvensis*. PloS one 8, e75820-e75820.

Larue, A.A.C., Raguso, R.A., Junker, R.R., 2016. Experimental manipulation of floral scent bouquets restructures flower-visitor interactions in the field. Journal of Animal Ecology 85, 396-408.

Lee, E.H., Tingey, D.T., Hogsett, W.E., 1988. Evaluation of ozone exposure indexes in exposure-response modeling. Environmental Pollution 53, 43-62.

Lei, H., Wuebbles, D.J., Liang, X.Z., 2012. Projected risk of high ozone episodes in 2050. Atmospheric Environment 59, 567-577.

Leisner, C.P., Ainsworth, E.A., 2012. Quantifying the effects of ozone on plant reproductive growth and development. Global Change Biology 18, 606-616.

Lemoine, N.P., Burkepile, D.E., Parker, J.D., 2014. Variable effects of temperature on insect herbivory. Peerj 2, 18.

Lenth, R., 2020a. emmeans: Estimated marginal means, aka least-squares means. R package version 1.4.7.

Lenth, R., 2020b. emmeans: Estimated marginal means, aka least-squares means. R package version 1.4.7.

Li, T., Blande, J.D., Holopainen, J.K., 2016. Atmospheric transformation of plant volatiles disrupts host plant finding. Scientific Reports 6, 10.

Lin, M.Y., Horowitz, L.W., Payton, R., Fiore, A.M., Tonnesen, G., 2017. US surface ozone trends and extremes from 1980 to 2014: quantifying the roles of rising Asian emissions, domestic controls, wildfires, and climate. Atmospheric Chemistry and Physics 17, 2943-2970.

Lin, M.Y., Horowitz, L.W., Xie, Y.Y., Paulot, F., Malyshev, S., Shevliakova, E., Finco, A., Gerosa, G., Kubistin, D., Pilegaard, K., 2020. Vegetation feedbacks during drought exacerbate ozone air pollution extremes in Europe. Nature Climate Change 10, 444-+.

Lu, X., Zhang, L., Wang, X.L., Gao, M., Li, K., Zhang, Y.Z., Yue, X., Zhang, Y.H., 2020. Rapid Increases in Warm-Season Surface Ozone and Resulting Health Impact in China Since 2013. Environmental Science & Technology Letters 7, 240-247.

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, 904-912.

Lyons, T.M., Barnes, J.D., 1998. Influence of plant age on ozone resistance in *Plantago major*. New Phytologist 138, 83-89.

Maenpaa, M., Riikonen, J., Kontunen-Soppela, S., Rousi, M., Oksanen, E., 2011. Vertical profiles reveal impact of ozone and temperature on carbon assimilation of Betula pendula and Populus tremula. Tree Physiology 31, 808-818.

Manisalidis, I., Stavropoulou, E., Stavropoulos, A., Bezirtzoglou, E., 2020. Environmental and Health Impacts of Air Pollution: A Review. Frontiers in Public Health 8, 13.

Manninen, A.M., Holopainen, T., Lyytikainen-Saarenmaa, P., Holopainen, J.K., 2000. The role of low-level ozone exposure and mycorrhizas in chemical quality and insect herbivore performance on Scots pine seedlings. Global Change Biology 6, 111-121.

Martinez-Ghersa, M.A., Menendez, A.I., Gundel, P.E., Folcia, A.M., Romero, A.M., Landesmann, J.B., Ventura, L., Ghersa, C.M., 2017. Legacy of historic ozone exposure on plant community and food web structure. PloS one 12, 14.

McClung, C.R., Lou, P., Hermand, V., Kim, J.A., 2016. The importance of ambient temperature to growth and the induction of flowering. Frontiers in Plant Science 7, 7.

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

Meireles, A., Giaouris, E., Simões, M., 2016. Alternative disinfection methods to chlorine for use in the fresh-cut industry. Food Research International 82, 71-85.

Memmott, J., Craze, P.G., Waser, N.M., Price, M.V., 2007. Global warming and the disruption of plant-pollinator interactions. Ecology Letters 10, 710-717.

Menser, H.A., Heggestad, H.E., Jeffrey, R.N., Street, O.E., 1963. Response of plants to air pollutants .1. Effects of ozone on tobacco plants preconditioned by light and temperature. Plant Physiology 38, 605-&.

Mills, G., Harmens, H., Hayes, F., Pleijel, H., Büker, P., González-Fernández, I., 2017. Chapter III: Mapping critical levels for vegetation, Manual for modelling and mapping critical loads & levels.

Mills, G., Harmens, H., Wagg, S., Sharps, K., Hayes, F., Fowler, D., Sutton, M., Davies, B., 2016. Ozone impacts on vegetation in a nitrogen enriched and changing climate. Environmental Pollution 208, 898-908.

Mills, G., Hayes, F., Simpson, D., Emberson, L., Norris, D., Harmens, H., Buker, P., 2011. Evidence of widespread effects of ozone on crops and (semi-)natural vegetation in Europe (1990-2006) in relation to AOT40-and flux-based risk maps. Global Change Biology 17, 592-613.

Mills, G., Sharps, K., Simpson, D., Pleijel, H., Frei, M., Burkey, K., Emberson, L., Uddling, J., Broberg, M., Feng, Z.Z., Kobayashi, K., Agrawal, M., 2018. Closing the global ozone yield gap: Quantification and cobenefits for multistress tolerance. Global Change Biology 24, 4869-4893.

Mofikoya, A.O., Kivimaenpaa, M., Blande, J.D., Holopainen, J.K., 2018. Ozone disrupts adsorption of Rhododendron tomentosum volatiles to neighbouring plant surfaces, but does not disturb herbivore repellency. Environmental Pollution 240, 775-780.

Mortensen, L., Jorgensen, H.E., 1996. Responses of spring wheat (*Triticum aestivum* L) to ozone produced by either electric discharge and dry air or by UV-lamps and ambient air. Environmental Pollution 93, 121-127.

Mulligan, G.A., Bailey, L.G., 1975. Biology of Canadian weeds .8. *Sinapis arvensis* L. Canadian Journal of Plant Science 55, 171-183.

Neufeld, H.S., Sullins, A., Sive, B.C., Lefohn, A.S., 2019. Spatial and temporal patterns of ozone at Great Smoky Mountains National Park and implications for plant responses. Atmospheric Environment: X 2, 100023.

Nuvolone, D., Petri, D., Voller, F., 2018. The effects of ozone on human health. Environmental Science and Pollution Research 25, 8074-8088.

Ollerton, J., Winfree, R., Tarrant, S., 2011. How many flowering plants are pollinated by animals? Oikos 120, 321-326.

Pandiselvam, R., Mayookha, V.P., Kothakota, A., Sharmila, L., Ramesh, S.V., Bharathi, C.P., Gomathy, K., Srikanth, V., 2020. Impact of ozone treatment on seed germination - a systematic review. Ozone-Science & Engineering 42, 331-346.

Pashalidou, F.G., Fatouros, N.E., Van Loon, J.J.A., Dicke, M., Gols, R., 2015a. Plant-mediated effects of butterfly egg deposition on subsequent caterpillar and pupal development, across different species of wild Brassicaceae. Ecological Entomology 40, 444-450.

Pashalidou, F.G., Frago, E., Griese, E., Poelman, E.H., van Loon, J.J.A., Dicke, M., Fatouros, N.E., 2015b. Early herbivore alert matters: plant-mediated effects of egg deposition on higher trophic levels benefit plant fitness. Ecology Letters 18, 927-936.

Pashalidou, F.G., Lambert, H., Peybernes, T., Mescher, M.C., De Moraes, C.M., 2020. Bumble bees damage plant leaves and accelerate flower production when pollen is scarce. Science 368, 881-+.

Pashalidou, F.G., Lucas-Barbosa, D., van Loon, J.J.A., Dicke, M., Fatouros, N.E., 2013. Phenotypic plasticity of plant response to herbivore eggs: effects on resistance to caterpillars and plant development. Ecology 94, 702-713.

Peltonen, P.A., Julkunen-Tiitto, R., Vapaavuori, E., Holopainen, J.K., 2006. Effects of elevated carbon dioxide and ozone on aphid oviposition preference and birch bud exudate phenolics. Global Change Biology 12, 1670-1679.

Peltonen, P.A., Vapaavuori, E., Heinonen, J., Julkunen-Tiitto, R., Holopainen, J.K., 2010. Do elevated atmospheric CO_2 and O_3 affect food quality and performance of folivorous insects on silver birch? Global Change Biology 16, 918-935.

Petanidou, T., 2005. Sugars in Mediterranean floral nectars: an ecological and evolutionary approach. Journal of Chemical Ecology 31, 1065-1088.

Pinto, D.M., Blande, J.D., Nykanen, 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, 683-694.

Pinto, D.M., Blande, J.D., Souza, S.R., Nerg, A.M., Holopainen, J.K., 2010. Plant Volatile Organic Compounds (VOCs) in ozone (O_3) polluted atmospheres: the ecological effects. Journal of Chemical Ecology 36, 22-34.

Pleijel, H., Danielsson, H., Gelang, J., Sild, E., Sellden, G., 1998. Growth stage dependence of the grain yield response to ozone in spring wheat (Triticum aestivum L.). Agriculture Ecosystems & Environment 70, 61-68.

Powney, G.D., Carvell, C., Edwards, M., Morris, R.K.A., Roy, H.E., Woodcock, B.A., Isaac, N.J.B., 2019. Widespread losses of pollinating insects in Britain. Nature Communications 10, 6.

Prieto-Benitez, S., Ruiz-Checa, R., Bermejo-Bermejo, V., Gonzalez-Fernandez, I., 2021. The Effects of Ozone on Visual Attraction Traits of Erodium paularense (Geraniaceae) Flowers: Modelled Perception by Insect Pollinators. Plants-Basel 10, 14.

R Core Team, 2017. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

R Core Team, 2020. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Rämö, K., Kanerva, T., Ojanpera, K., Manninen, S., 2007. Growth onset, senescence, and reproductive development of meadow species in mesocosms exposed to elevated O_3 and CO_2. Environmental Pollution 145, 850-860.

Rankenberg, T., Geldhof, B., van Veen, H., Holsteens, K., Van de Poel, B., Sasidharan, R., 2021. Age-dependent abiotic stress resilience in plants. Trends in Plant Science 26, 692-705.

Reiling, K., Davison, A.W., 1992. Effects of a short ozone exposure given at different stages in the development of *Plantago major* L. New Phytologist 121, 643-647.

Reiling, K., Davison, A.W., 1994. Effects of exposure to ozone at different stages in the development of *Plantago major* L. on chlorophyll fluorescence and gas-exchange. New Phytologist 128, 509-514.

Riffell, J.A., Shlizerman, E., Sanders, E., Abrell, L., Medina, B., Hinterwirth, A.J., Kutz, J.N., 2014. Flower discrimination by pollinators in a dynamic chemical environment. Science 344, 1515-1518.

Rusman, Q., Lucas-Barbosa, D., Poelman, E.H., 2018. Dealing with mutualists and antagonists: Specificity of plant-mediated interactions between herbivores and flower visitors, and consequences for plant fitness. Functional Ecology 00, 1-14.

Rusman, Q., Lucas-Barbosa, D., Poelman, E.H., Dicke, M., 2019a. Ecology of Plastic Flowers. Trends in Plant Science 24, 725-740.

Rusman, Q., Poelman, E.H., Nowrin, F., Polder, G., Lucas-Barbosa, D., 2019b. Floral plasticity: Herbivore-species-specific-induced changes in flower traits with contrasting effects on pollinator visitation. Plant Cell and Environment 42, 1882-1896.

Saunier, A., Blande, J.D., 2019. The effect of elevated ozone on floral chemistry of Brassicaceae species. Environmental Pollution 255, 10.

Schenk, M., Krauss, J., Holzschuh, A., 2018. Desynchronizations in bee-plant interactions cause severe fitness losses in solitary bees. Journal of Animal Ecology 87, 139-149.

Schiestl, F.P., 2015. Ecology and evolution of floral volatile-mediated information transfer in plants. New Phytologist 206, 571-577.

Sicard, P., De Marco, A., Agathokleous, E., Feng, Z.Z., Xu, X.B., Paoletti, E., Rodriguez, J.J.D., Calatayud, V., 2020. Amplified ozone pollution in cities during the COVID-19 lockdown. Science of The Total Environment 735, 10.

Singh, A.A., Agrawal, S.B., Shahi, J.P., Agrawal, M., 2014. Assessment of growth and yield losses in two Zea mays L. cultivars (quality protein maize and nonquality protein maize) under projected levels of ozone. Environmental Science and Pollution Research 21, 2628-2641.

Smallegange, R.C., van Loon, J.J.A., Blatt, S.E., Harvey, J.A., Agerbirk, N., Dicke, M., 2007. Flower vs. leaf feeding by pieris brassicae: glucosinolate-rich flower tissues are preferred and sustain higher growth rate. Journal of Chemical Ecology 33, 1831-1844.

Stabler, D., 2016. Impacts of ozone pollution on nectar and pollen quality and their significance for pollinators (Ph.D. Thesis), School of Biology. Newcastle University, Newcastle, UK.

Takeno, K., 2016. Stress-induced flowering: the third category of flowering response. Journal of Experimental Botany 67, 4925-4934.

Telesnicki, M.C., Martinez-Ghersa, M.A., Ghersa, C.M., 2018. Plant oxidative status under ozone pollution as predictor for aphid population growth: The case of Metopolophium dirhodum (Hemiptera: Aphididae) in Triticum aestivum (Poales: Poaceae). Biochemical Systematics and Ecology 77, 51-56.

Thackeray, S.J., Henrys, P.A., Hemming, D., Bell, J.R., Botham, M.S., Burthe, S., Helaouet, P., Johns, D.G., Jones, I.D., Leech, D.I., Mackay, E.B., Massimino, D., Atkinson, S., Bacon, P.J., Brereton, T.M., Carvalho, L., Clutton-Brock, T.H., Duck, C., Edwards, M., Elliott, J.M., Hall, S.J.G., Harrington, R., Pearce-Higgins, J.W., Hoye, T.T., Kruuk, L.E.B., Pemberton, J.M., Sparks, T.H., Thompson, P.M., White, I., Winfield, I.J., Wanless, S., 2016. Phenological sensitivity to climate across taxa and trophic levels. Nature 535, 241-U294.

The Chinese State Council, 2013. The Airborne Pollution Prevention and Control Plan (2013-2017).

Van Dingenen, R., Dentener, F.J., Raes, F., Krol, M.C., Emberson, L., Cofala, J., 2009. The global impact of ozone on agricultural crop yields under current and future air quality legislation. Atmospheric Environment 43, 604-618.

Vanbergen, A.J., Baude, M., Biesmeijer, J.C., Britton, N.F., Brown, M.J.F., Brown, M., Bryden, J., Budge, G.E., Bull, J.C., Carvell, C., Challinor, A.J., Connolly, C.N., Evans, D.J., Feil, E.J., Garratt, M.P., Greco, M.K., Heard, M.S., Jansen, V.A.A., Keeling, M.J., Kunis, W.E., Marris, G.C., Memmott, J., Murray, J.T., Nicolson, S.W., Osborne, J.L., Paxton, R.J., Pirk, C.W.W., Polce, C., Potts, S.G., Priest, N.K., Raine, N.E., Roberts, S., Ryabov, E.V., Shafir, S., Shirley, M.D.F., Simpson, S.J., Stevenson, P.C., Stone, G.N., Termansen, M., Wright, G.A., Insect Pollinators, I., 2013. Threats to an ecosystem service: pressures on pollinators. Frontiers in Ecology and the Environment 11, 251-259.

Vanderplanck, M., Lapeyre, B., Brondani, M., Opsommer, M., Dufay, M., Hossaert-McKey, M., Proffit, M., 2021. Ozone Pollution Alters Olfaction and Behavior of Pollinators. Antioxidants 10, 16.

Visser, M.E., Gienapp, P., 2019. Evolutionary and demographic consequences of phenological mismatches. Nature Ecology & Evolution 3, 879-885.

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, 305-311.

Wang, P., Marsh, E.L., Ainsworth, E.A., Leakey, A.D.B., Sheflin, A.M., Schachtman, D.P., 2017. Shifts in microbial communities in soil, rhizosphere and roots of two major crop systems under elevated CO_2 and O_3. Scientific Reports 7, 12.

Warwick, S.I., Beckie, H.J., Thomas, A.G., McDonald, T., 2000. The biology of Canadian weeds. 8. *Sinapis arvensis* L. (updated). Canadian Journal of Plant Science 80, 939-961.

Webber, S.M., Garratt, M.P.D., Lukac, M., Bailey, A.P., Huxley, T., Potts, S.G., 2020. Quantifying crop pollinator-dependence and pollination deficits: The effects of experimental scale on yield and quality assessments. Agriculture Ecosystems & Environment 304, 8.

Wedlich, K.V., Rintoul, N., Peacock, S., Cape, J.N., Coyle, M., Toet, S., Barnes, J., Ashmore, M., 2012. Effects of ozone on species composition in an upland grassland. Oecologia 168, 1137-1146.

Wood, S.N., 2003. Thin plate regression splines. Journal of the Royal Statistical Society Series B-Statistical Methodology 65, 95-114.

Yan, Y.Y., Lin, J.T., Pozzer, A., Kong, S.F., Lelieveld, J., 2019. Trend reversal from high-to-low and from rural-to-urban ozone concentrations over Europe. Atmospheric Environment 213, 25-36.

Yan, Y.Y., Pozzer, A., Ojha, N., Lin, J.T., Lelieveld, J., 2018. Analysis of European ozone trends in the period 1995-2014. Atmospheric Chemistry and Physics 18, 5589-5605.

Yeung, L.Y., Murray, L.T., Martinerie, P., Witrant, E., Hu, H., Banerjee, A., Orsi, A., Chappellaz, J., 2019. Isotopic constraint on the twentieth-century increase in tropospheric ozone. Nature 570, 224-227.

Young, P.J., Archibald, A.T., Bowman, K.W., Lamarque, J.F., Naik, V., Stevenson, D.S., Tilmes, S., Voulgarakis, A., Wild, O., Bergmann, D., Cameron-Smith, P., Cionni, I., Collins, W.J., Dalsoren, S.B., Doherty, R.M., Eyring, V., Faluvegi, G., Horowitz, L.W., Josse, B., Lee, Y.H., MacKenzie, I.A., Nagashima, T., Plummer, D.A., Righi, M., Rumbold, S.T., Skeie, R.B., Shindell, D.T., Strode, S.A., Sudo, K., Szopa, S., Zeng, G., 2013. Pre-industrial to end 21st century projections of tropospheric ozone from the Atmospheric Chemistry and Climate Model Intercomparison Project (ACCMIP). Atmospheric Chemistry and Physics 13, 2063-2090.

Ziemke, J.R., Oman, L.D., Strode, S.A., Douglass, A.R., Olsen, M.A., McPeters, R.D., Bhartia, P.K., Froidevaux, L., Labow, G.J., Witte, J.C., Thompson, A.M., Haffner, D.P., Kramarova, N.A., Frith, S.M., Huang, L.K., Jaross, G.R., Seftor, C.J., Deland, M.T., Taylor, S.L., 2019. Trends in global tropospheric ozone inferred from a composite record of TOMS/OMI/MLS/OMPS satellite measurements and the MERRA-2 GMI simulation. Atmospheric Chemistry and Physics 19, 3257-3269.

Zinta, G., Khan, A., AbdElgawad, H., Verma, V., Srivastava, A.K., 2016. Unveiling the redox control of plant reproductive development during abiotic stress. Frontiers in Plant Science 7, 6.

Publication list

Publications that integrate the doctoral dissertation

Peer-reviewed publications

Duque L., Poelman E.H., Steffan-Dewenter I., **2021**, *Plant age at the time of ozone exposure affects flowering patterns, biotic interactions and reproduction of wild mustard*. Scientific Reports 11, 10

Duque L., Poelman E.H., Steffan-Dewenter I., **2021**, *Effects of ozone stress on flowering phenology, plant-pollinator interactions and plant reproductive success*. Environmental Pollution 272, 115953.

Duque L., Poelman E.H., Steffan-Dewenter I., **2019**, *Plant-mediated effects of ozone on herbivores depend on exposure duration and temperature*, Scientific Reports, 9 (1), 19891.

Previous publications

Peer-reviewed publications

Miranda, A. I., Ferreira J., Silveira C., Relvas H., **Duque L.**, Roebeling P., Lopes M., Costa S., Monteiro A., Gama C., Sá E., Borrego C. and Teixeira J. P., **2016**, *A Cost-Efficiency and Health Benefit Approach to Improve Urban Air Quality*, Science of The Total Environment, 569, 342-51.

Duque L., Relvas H., Silveira C., Ferreira J., Monteiro A., Gama C., Rafael S., Freitas S., Borrego C., Miranda A.I., **2016**, *Evaluating strategies to reduce urban air pollution*, Atmospheric Environment, 127, 196-204

Ribeiro H., Guimarães F., **Duque L.**, Noronha F., Abreu I., **2015**, *Characterisation of particulate matter on airborne pollen grains*, Environmental Pollution, 206, 7-16

Ribeiro H., **Duque L.**, Sousa R., Cruz A., Gomes C. R., Esteves da Silva J. C. G., Abreu I., **2014**, *Changes in the IgE-reacting protein profiles of Acer negundo, Platanus x acerifolia and Quercus robur pollen in response to ozone treatment*, International Journal of Environmental Health Research, 24 (6), 515-527

Sousa R., Ribeiro H., Cruz A., **Duque L.**, Abreu I., **2014**, *Parietaria judaica pollen: Aerobiology and allergenicity*, Revista Portuguesa de Imunoalergologia, 22 (2), 81-92

Sousa R., Osorio H., **Duque L.**, Ribeiro H., Cruz A., Abreu I., **2014**, *Identification of Plantago lanceolata pollen allergens using an immunoproteomic approach*, Journal of Investigational Allergology and Clinical Immunology, 24 (3), 177-183

Duque L., Guimarães F., Ribeiro H., Sousa R., Abreu I., **2013**, *Elemental characterization of the airborne pollen surface using Electron Probe Microanalysis (EPMA)*, Atmospheric Environment, 75, 296-302

Ribeiro H., **Duque L.**, Sousa R., Abreu I., **2013**, *Ozone effects on soluble protein of Acer negundo, Quercus robur and Platanus spp. pollen*, Aerobiologia, 29, 443-447

Guimarães F., **Duque L.**, Ribeiro H., Sousa R., Abreu I., **2012**, *Contribution of EPMA to airborne pollen analysis*, IOP Conference Series: Materials Science and Engineering, 32 (1)

Sousa R., **Duque L.**, Duarte A. J., Gomes C. R., Ribeiro H., Cruz A., Esteves da Silva J. C. G., Abreu I., **2012**, *In vitro exposure of Acer negundo pollen to atmospheric levels of* SO_2 *and* NO_2*: effects on allergenicity and germination*, Environmental Science and Technology, 46 (4), 2406-2412

Other Publications

Relvas H., Miranda A.I., Turrini E., Lopes D., Silveira C., Ferreira J., Lopes M., Sá E., **Duque L.**, Borrego C., Volta M., **2016**, *Chapter 26- Air quality modelling to support decision-making: scenario and optimization approaches* in Air modelling and its application XXIV, Springer proceedings in complexity, 161-166

Duque L., Ribeiro H., Gomes C. R., Esteves da Silva J. C. G., Abreu I., **2013**, *Germination rate of three anemophilous pollen types after in vitro exposure to gaseous atmospheric pollutants*, 1st Iberian Meeting on Aerosol Science and Technology – Proceedings Book, 163-166

Duque L., Guimarães F., Ribeiro H., Sousa R., Abreu I., **2012**, *Airborne Platanus pollen analysis by EPMA*, Microscopy and Microanalysis, 18 (Suppl 5), 39-40

Sousa, R., **Duque, L.**, Ribeiro, H., Abreu, I., Duarte, A., Gomes, C., Cruz, A., Esteves da Silva, J., **2012**, *Effects of two atmospheric pollutants (*SO_2 *and* NO_2*) on protein*

content and allergenic properties of Acer negundo L. pollen. Allergy, 67 (Supl. 96), 574-575

Duque L., *Dinâmicas de colheita de pólen e actividade polinizadora de Apis mellifera em Cucumis melo (Pollen-foraging dynamics and pollinating activity of Apis mellifera in Cucumis melo)*, Master's dissertation. **February 2010**, Faculty of Sciences, University of Porto, Portugal.