Chemical Ecology of Interactions between Scots Pine (*Pinus sylvestris*) and the Herbivorous Sawfly *Diprion pini*: Effects of Sawfly Pheromones and Pine Needle Age

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To my beloved wife, Raha, whose love and support have been my greatest inspiration....

Declaration of Authorship

I hereby declare that I am solely responsible for the content of my doctoral dissertation titled "Chemical Ecology of Interactions between Scots Pine (*Pinus sylvestris*) and the Herbivorous Sawfly *Diprion pini*: Effects of Sawfly Pheromones and Pine Needle Age" and that I have used only the sources and references cited within the dissertation.

Berlin, February 2025

Md Asifur Rahman Soad

This thesis is based on the following manuscripts:

1) **Rahman-Soad, A., Bittner, N., & Hilker, M. (2024). Pine response to sawfly pheromones: Effects on sawfly's oviposition and larval growth. *Insects, 15*(6), 458. https://doi.org/10.3390/insects15060458**

Author contributions:

Conceptualization, A.R.-S. and M.H.; methodology, A.R.-S. and M.H.; software, A.R.-S.; validation, A.R.-S.; formal analysis, A.R.-S.; investigation, A.R.-S. and N.B. (**Figure 1**); resources, A.R.-S. and M.H.; data analysis, A.R.-S.; writing—original draft preparation, A.R.-S.; writing—review and editing, A.R.-S. and M.H.; visualization, A.R.-S.; supervision, M.H.; project administration, M.H.; funding acquisition, M.H. All authors have read and agreed to the published version of the manuscript.

2) **Rahman-Soad, A., Skuras, L., Reinecke, A., Varama, M., & Hilker, M. (2024). Sawfly sex pheromones: Analysis of their impact on pine odor attractive to egg parasitoids. *J Chem Ecol, 50*(6), 620–630. https://doi.org/10.1007/s10886-024-01547-1**

Author contributions:

ARS and MH conceived and designed the study. ARS treated the plants with pheromone and insects. MV collected the egg parasitoids. LS and ARS performed the olfactometer bioassays. ARS conducted the headspace sampling from trees. LS, ARS, and AR evaluated the GC-MS data. ARS conducted the statistical analyses. ARS wrote the first draft of the manuscript, ARS and MH wrote revised versions, and all authors contributed to the final version of the manuscript.

3) **Rahman-Soad, A., Krause, S., Hagemann, L., & Hilker, M. (manuscript submitted in Nov. 2024, currently in revision). Needle age-dependent defence of Scots pine against insect herbivory.**

Author contributions:

A.R.S. and M.H. conceptualised the study and designed the experiments. A.R.S. carried out the oviposition preference and egg survival experiments. S.K. carried out all experiments with larvae and prepared samples for phytohormone extraction and analysis. L.H. performed the phytohormone extraction. S.K. and A.R.S. conducted protein extraction and measured PI activity. A.R.S. conducted the statistical analyses. A.R.S. wrote the first draft of the manuscript, A.R.S. and M.H. wrote revised versions, and all authors contributed to the final version of the manuscript.

Contents

Summary

This thesis explores the influence of sawfly sex pheromones and the role of needle age in shaping pine defences against sawfly infestation. The research is centred on how pine trees respond to sex pheromones emitted by sawflies and how these responses modulate both direct and indirect pine defences against sawflies. Furthermore, it delves into how the age of pine needles affects their susceptibility to sawfly attack. All studies were conducted with young Scots pine trees (*Pinus sylvestris*) and the sawfly *Diprion pini* in the laboratory. Larvae of this sawfly species gregariously feed upon pine needles and may heavily damage pine forests.

The first major focus of the experimental part of this thesis was the investigation of the effect of *D. pini* sex pheromones on pine direct defences. Previous studies have shown that responses of Scots pine to these sex pheromones result in enhanced accumulation of hydrogen peroxide in pine needles and subsequently to reduced *D. pini* egg survival rates. Hence, exposure of pine to the pheromones enhances (primes) pine defences against sawfly eggs.

- In the study here, we first addressed the question whether *D. pini* females evolved a counter-adaptation to the pheromone-mediated Scots pine defence against sawfly eggs by avoiding pheromone-exposed pine trees for oviposition. The bioassays showed that the sawfly females cannot discriminate between the odour of pheromone-exposed and unexposed pine trees. However, upon contact with pine trees, *D. pini* females preferred oviposition on unexposed trees and were reluctant to lay their eggs on the pheromone-exposed trees.
- We further studied whether the improved defensive effects of pheromone-exposed Scots pine on sawfly eggs also extend to defence against sawfly larvae hatching from survived eggs. However, exposure of pine to sawfly sex pheromones did not affect the weight of larvae nor their pupation success.
- Thus, pine responses to *D. pini* sex pheromones affect (enhance) the defence against the initial step of sawfly infestation, i.e. the egg deposition, but not the defence against later insect developmental stages. The results are discussed with respect to two well known hypotheses in entomology, i.e. the “mother knows best” hypothesis and “oviposition preference – insect performance” hypothesis.

The second focus of the experimental part of this thesis addressed the question whether the pheromone-mediated priming of pine responses is restricted to direct defences against sawfly eggs or if it also enhances indirect defences of pine trees against eggs. We first examined whether (i) exposure of egg-free *P. sylvestris* trees to *D. pini* sex pheromones renders pine attractive to egg parasitoids of *D. pini*, and/or (ii) whether exposure of pine to first the pheromones and subsequently to *D. pini* egg deposition can prime the known egg-induced emission of (*E*)-β-farnesene from pine needles. This sesquiterpene has been shown in previous studies to attract egg parasitoids when combined with four other non-induced pine terpenoids.

- Our study revealed that the egg parasitoid *Closterocerus ruforum* did not distinguish between the odour from pheromone-exposed and unexposed trees without sawfly eggs.
- Chemical analysis of pine volatile profiles by coupled gas chromatography - mass spectrometry further showed that pheromone exposure did not significantly change the emission of (*E*)-β-farnesene from pine needles, regardless of the presence of eggs. However, as demonstrated in previous analyses of the odour of egg-laden branches of mature pines, sawfly egg deposition induced the emission of (*E*)-β-farnesene also in the young pine trees used in the studies of this thesis. Furthermore, the chemical analysis revealed that *D. pini* egg deposition on the young

pines additionally induced the emission rate of (*Z*)-β-ocimene, one of the four terpenes relevant for attracting *C. ruforum* when combined with enhanced emission rates of (*E*)-β-farnesene.

- The results are discussed in the context of previous knowledge of this tripartite interaction (*P. sylvestris – D. pini – C. ruforum*), considering the potential trade-offs between direct and indirect defence due to resource allocation costs.

The third experimental part of the thesis addressed the question of how the age of *P. sylvestris* needles influences their defences against *D. pini* larval herbivory. It is well known that juvenile foliage of angiosperms shows different responses to stress than a few months older, mature foliage. However, much less is known about age-dependent stress responses of conifer needles, which may become several years old. To address this gap in knowledge, proteinase inhibitor (PI) activities and phytohormone concentrations (jasmonic acid, jasmonic acid-isoleucine, salicylic acid, and abscisic acid) of current-year (young) and previous-year (old) pine needles were analysed; untreated needles of these ages and needles damaged by *D. pini* larvae were used. The study further tested how the insect responds to pine foliage of different age. Thus, we could elucidate how a pine-specialised insect copes with age-dependent needle defences.

- The results of this third part showed that young and old pine needles responded to larval feeding damage similarly with respect to the damage-induced increase of jasmonic acid and jasmonic acid-isoleucine concentrations. However, levels of salicylic acid and abscisic acid were induced by sawfly feeding damage to a higher level in young than old pine needles. PI activities were constitutively higher in young needles, but were not significantly inducible upon sawfly feeding, neither in young nor old needles.
- Sawfly females preferred to oviposit on old needles, where larvae gained most weight.
- This study highlights the plasticity of pine defence traits across different needle ages. The results show some differences between age-dependent foliage responses of angiosperms and pine to herbivory, but also similarities. Moreover, our study elucidates the ability of a pine-specialised herbivore to adapt to defences of its host. Our results are consistent with a long-studied hypothesis in entomology ("oviposition preference – insect performance" hypothesis) as well as with the "optimal plant defence theory", according to which young foliage is particularly valuable.

In conclusion, the results of this thesis offer significant novel insights into the chemical ecology of Scots pine and its interactions with herbivorous sawflies. The general introduction outlines the state of the art on (priming of) plant defences against insect infestation prior to this thesis. The general discussion focuses on the mechanisms by which plants perceive volatile organic compounds and further addresses pheromone-mediated priming and the influence of leaf and plant age on anti-herbivore defence. The results of this thesis may contribute to a deeper understanding of plant - insect interactions.

Zusammenfassung

Diese Doktorarbeit untersuchte den Einfluss von Sexualpheromonen einer Blattwespe auf die Abwehr der Waldkiefer gegen Blattwespenbefall. Die Forschung konzentrierte sich darauf, wie Kiefern auf Pheromone der Blattwespen reagieren und wie diese Reaktionen sowohl die direkte als auch die indirekte Abwehr gegen Blattwespenbefall modulieren. Darüber hinaus wurde untersucht, wie das Alter der Kiefernnadeln deren Anfälligkeit für Blattwespenbefall beeinflusst. Alle Untersuchungen wurden im Labor mit jungen Waldkiefern (*Pinus sylvestris*) und der Kiefernbuschhornblattwespe *Diprion pini* durchgeführt. Die Larven dieser Blattwespenart fressen gregär an Kiefernnadeln und können Kiefernwälder erheblich schädigen.

Der erste Schwerpunkt dieser Arbeit lag auf Untersuchungen zur Wirkung von *D. pini* Sexualpheromonen auf die direkten Abwehrmechanismen der Kiefer. Frühere Studien haben gezeigt, dass die Reaktionen der Waldkiefer auf diese Pheromone zu einer verstärkten Konzentration von Wasserstoffperoxid in Kiefernnadeln und in der Folge zu einer reduzierten Überlebensrate der *D. pini* Eier führen. Demnach verstärkt die Pheromon-Exposition von Kiefern die Abwehr gegen Blattwespeneier.

- In der hier vorliegenden Studie wurde zunächst untersucht, ob *D. pini* Weibchen eine Gegenanpassung an die Pheromon-vermittelte Kiefernabwehr entwickelt haben, indem sie Pheromon-exponierte Kiefern für die Eiablage meiden. Die Biotests zeigten, dass Blattwespenweibchen den Geruch von Pheromon-exponierten und nicht-exponierten Kiefern nicht unterscheiden können. Bei Kontakt mit den Kiefern bevorzugten sie jedoch die Eiablage auf nicht-exponierten Bäumen und waren zögerlich, ihre Eier auf zuvor Pheromon-exponierten Bäumen abzulegen.
- Ferner wurde untersucht, ob sich die verbesserte Abwehr Pheromon-exponierter Waldkiefern gegen Eier auch auf die Abwehr gegen Blattwespenlarven erstreckt, die aus überlebenden Eiern schlüpfen. Die Pheromon-Exposition der Kiefer nahm keinen Einfluss auf das Gewicht der Larven und deren Verpuppungserfolg.
- Somit haben die Reaktionen der Kiefer auf *D. pini* Sexualpheromone zwar einen verstärkenden Effekt auf die Abwehr gegen den ersten Schritt des Blattwespenbefalls, nämlich die Eiablage, aber nicht auf die Abwehr gegen spätere Entwicklungsstadien des Insekts. Die Ergebnisse werden im Zusammenhang mit der in der Entomologie gut bekannten „mother knows best"-Hypothese und der Hypothese zur „oviposition preference – insect performance" diskutiert.

Der zweite Schwerpunkt dieser Arbeit lag auf der Frage, ob die Pheromon-vermittelte Prägung der Kieferabwehr beschränkt ist auf direkte Abwehr gegen Blattwespeneier oder ob sie auch indirekte Abwehr der Waldkiefer gegen Eier verstärkt. Wir haben geprüft, ob (i) die Pheromon-Exposition von Ei-freien Kiefern die Bäume attraktiv macht für Eiparasitoide von *D. pini* und/oder (ii) ob die Pheromon-Exposition der Kiefern mit nachfolgender Eiablage auch die (bereits bekannte) Eiablage-induzierte Emission von (*E*)-β-Farnesen aus den Kiefernnadeln prägen (verstärken) kann. Für dieses Sesquiterpen ist bekannt, dass es in Kombination mit vier anderen nicht-induzierten Terpenen der Kiefernadeln die Eiparasitoide anlockt.

- Unsere Studie zeigte, dass der Eiparasitoid *Closterocerus ruforum* nicht zwischen dem Duft von Pheromon-exponierten und nicht-exponierten Bäumen ohne Blattwespeneier unterscheiden konnte.
- Chemische Analysen der Duftprofile mittels gekoppelter Gaschromatographie-Massenspektrometrie ergaben, dass eine Pheromon-Exposition die Emission von (*E*)-β-Farnesen nicht signifikant veränderte, weder in Waldkiefern mit Blattwespeneiern noch in

solchen ohne Eier. Wie jedoch bereits in früheren Duftanalysen von Eier-belegten Zweigen älterer Kiefernbäume gezeigt worden war, induzierte die Eiablage von Blattwespen die Freisetzung von (*E*)-β-Farnesen auch in den hier untersuchten jungen Waldkiefern. Darüber hinaus zeigte sich, dass die Eiablage von *D. pini* auf den jungen Kiefern zusätzlich die Emissionsrate von (*Z*)-β-Ocimen erhöhte. (*Z*)-β-Ocimen ist eines der vier Terpene, die in Kombination mit erhöhten Emissionsraten von (*E*)-β-Farnesen für die Anlockung von *C. ruforum* relevant sind.

- Die Ergebnisse werden im Zusammenhang mit dem bisherigen Wissen über diese tritrophische Interaktion (*P. sylvestris – D. pini – C. ruforum*) diskutiert; dabei werden unter anderem die potenziellen *trade-offs* zwischen direkter und indirekter Abwehr aufgrund von Ressourcenallokationskosten beleuchtet.

Der dritte Teil der experimentellen Arbeiten befasste sich mit der Frage, wie das Alter der *P. sylvestris* Nadeln deren Abwehr gegen Fraß durch Blattwespenlarven beeinflusst. Junge Blätter von Angiospermen reagieren bekanntlich anders auf Stress als einige Monate ältere, reife Blätter. Weniger bekannt ist jedoch über die Altersabhängigkeit der Stressreaktionen von Koniferennadeln, die mehrere Jahre alt werden können. Diese Wissenslücke wurde adressiert, indem Proteinase-Inhibitor (PI) Aktivitäten und Phytohormonkonzentrationen (Jasmonsäure, Jasmonsäure-Isoleucin, Salicylsäure und Abscisinsäure) in jungen (diesjährigen) und alten (vorjährigen) Kiefernadeln analysiert wurden; dabei wurden unbehandelte Nadeln dieser Altersgruppen und Nadeln, die durch *D. pini* Larvalfraß geschädigt waren, untersucht. Diese Studie testete auch, wie die Blattwespen auf Nadeln unterschiedlichen Alters reagieren. So wurde beleuchtet, wie ein auf Kiefernbäume spezialisiertes Insekt mit altersabhängigen Abwehrreaktionen der Kiefernnadeln umgeht.

- Die Ergebnisse dieses dritten Teils der Arbeit zeigten, dass junge und alte Kiefernnadeln auf Larvalfraß ähnlich reagierten, wenn man den fraßinduzierten Anstieg der Jasmonsäure(-Isoleucin)-Konzentration betrachtet. Die Konzentrationen von Salicylsäure und Abscisinsäure waren durch Larvalfraß in jungen Nadeln allerdings stärker induziert als in alten. Die PI Aktivitäten waren konstitutiv höher in jungen Nadeln, aber sie waren nicht signifikant induzierbar durch Larvalfraß, weder in alten noch in jungen Nadeln.
- Die Blattwespenweibchen bevorzugten zur Eiablage die alten Nadeln, an denen die Larven auch das meiste Gewicht erzielten.
- Diese Studie zeigt deutlich, wie plastisch einige Merkmale der Abwehr der Waldkiefer über verschiedene Nadelalter hinweg sind. Es zeigten sich einige Unterschiede, aber auch Gemeinsamkeiten zwischen den blattaltersabhängigen Reaktionen von Angiospermen und Kiefern auf Herbivorie. Darüber hinaus verdeutlicht die Studie die Fähigkeit eines auf Kiefern spezialisierten Herbivoren, sich an Abwehrmechanismen des Wirtes anzupassen. Die Ergebnisse stimmen sowohl mit einer langjährigen Hypothese in der Entomologie („oviposition preference – insect performance“ Hypothese) als auch mit der Theorie der optimalen Pflanzenabwehr überein, wonach junge Blätter besonders wertvoll sind.

Zusammenfassend bieten die Untersuchungen dieser Doktorarbeit aufschlußreiche, neue Einblicke in die chemische Ökologie der Interaktionen zwischen Waldkiefer und herbivoren Blattwespen. In der allgemeinen Einleitung wird der vor dieser Doktorarbeit bekannte Stand der Forschung über pflanzliche Abwehr gegen Insektenbefall und über Prägung (*Priming*) dieser Abwehr skizziert. Die allgemeine Diskussion befasst sich mit möglichen Mechanismen, mit denen Pflanzen flüchtige organische Verbindungen wahrnehmen, sowie mit Pheromon-vermitteltem *Priming* und dem Einfluss des Blatt- und Pflanzenalters auf die Abwehr. Die Resultate dieser Arbeit können einen Beitrag zu einem tieferen Verständnis von Pflanzen-Insekten Interaktionen leisten.

Chapter 1: General Introduction

1.1 Chemical Ecology of Plant-Insect Interactions

1.1. 1 Direct and indirect chemical plant defence mechanisms

Plants and herbivores have been interacting for millions of years. Over time, plants have evolved numerous mechanisms to defend themselves against attacks by herbivores (Wu & Baldwin, 2010). The plethora of different chemical defences in plants providing a major barrier to herbivory can be classified as constitutive and inducible defences (Mithöfer et al., 2008; Mithöfer & Boland, 2012).

Plants employ a diverse array of constitutive chemical defences against herbivores, including alkaloids, glucosinolates, terpenoids, and proteinase inhibitors (Arnason & Bernards, 2010; Mithöfer & Boland, 2012). These compounds can be toxic, repellent, or anti- nutritive to herbivores, acting through various mechanisms such as membrane disturbance and metabolic inhibition (Mithöfer & Boland, 2012). They may act against herbivory upon roots and foliage, whereby belowground herbivory may affect aboveground defences, and vice versa (Kaplan et al., 2008). The maintenance of constitutive defences has costs, which may have negative impact on growth and reproduction (Endara & Coley, 2011; Endara et al., 2023).

In contrast, inducible defences are activated after herbivore or pathogen attack and are regulated by a signalling network triggered by the plant's perception machinery (Wu & Baldwin, 2009). The ability to mobilise defences on demand allows a plant to avoid unnecessary investment in defence traits, especially in environments where antagonists are rare (Walling, 2000; Karban, 2011). The complex signalling web involved in these responses includes several phytohormones, among them particularly jasmonic acid, salicylic acid, abscisic acid and ethylene (Pieterse et al., 2006; Bari & Jones, 2009). Herbivory also triggers large-scale, species-specific transcriptional changes, necessary for encoding the enzymes involved in efficient defence signalling (Wu & Baldwin, 2009).

Both constitutive and inducible defences can further be classified into direct and indirect defences (Heil & Baldwin, 2002; Cipollini et al., 2003; Mithöfer et al., 2008). These defences can act against egg-laying and feeding insect herbivores.

Direct defences in plants against feeding insects include the production of anti-nutritive and toxic compounds that directly affect herbivores (Gatehouse, 2002; Chen, 2008; War et al., 2012). Anti-nutritive defences may act already prior to ingestion of leaf material by the presence of repellent or feeding deterring compounds. However, plants can also produce a wide array of compounds impairing digestion of plant material, and thus nutrition. The numerous known toxic plant compounds interact with a wide range of insect (cellular) targets, thus directly harming the herbivore or killing it (Chen, 2008). A complex array of specific defence mechanisms has been detected in the numerous interactions between plant and insect species. The major defensive chemicals that have been characterised in various plant - insect interactions include numerous low molecular plant secondary metabolites as well as proteases, lectins, amino acid deaminases, polyphenol oxidases, and other defensive proteins (Chen, 2008; Fürstenberg-Hägg et al., 2013; Wari et al., 2022). These directly defensive compounds are known to be present

constitutively in plants, but numerous of them have also been shown to increase their concentration and activity upon induction by herbivory.

Plants are not only adept at defending themselves against feeding insects but are also highly effective in protecting themselves by direct defence against insect eggs. Some plants show constitutive defensive devices against insect egg deposition, such as oviposition-deterring leaf volatiles (Schoonhoven et al., 2005, Braccini et al., 2015). During the last two decades, numerous egg-inducible, direct defences against insect eggs have been detected. In response to insect egg deposition, some plants form neoplasms, which are abnormal growths that can cause egg detachment or egg desiccation (Doss et al., 2000). For example, in pea pods, bruchid beetle eggs induce strong gene upregulation, leading to neoplasm formation (Doss et al., 2000). In rice plants, the egg-induced production of ovicidal compounds directly kills the eggs (Seino et al., 1996). Another direct defence involves the egg-induced growth of new tissue around the eggs, which can crush them (Desurmont et al., 2021). Furthermore, plants can accumulate reactive oxygen species (ROS) at the site of egg deposition, leading to necrotic tissue formation that detaches or desiccates the eggs (Bittner et al., 2017). This hypersensitive-like response (HR) to eggs is observed in several plant species and is often linked with the upregulation of pathogenesis-related (PR) genes, such as *PR1* and *PR5*, which are also induced by insect eggs (Little et al., 2007; Fatouros et al., 2016; Griese et al., 2021). Studies have shown that PR-encoding genes are upregulated in response to eggs from various insect species across different plants, including *Arabidopsis thaliana* and *Brassica nigra* (Little et al., 2007; Griese et al., 2021).

Indirect defences in plants involve attraction of natural enemies of egg-laying or feeding herbivores. Again, these defensive devices may be present constitutively or be induced by infestation. A well-known constitutive, indirect defence against herbivores are the hollow thorns of acacia; these thorns provide shelter for predatory ants, which serve as “bodyguards” of the plants and feed upon herbivorous insects that attack the plant (Heil & McKey, 2003; Hilker & Fatouros, 2015; Hundacker et al., 2022; Palmer, 2023). The mode of indirect, induced defence acting via the egg- or feeding-induced release of volatile organic compounds (VOCs) has been intensively studied. The induced plant volatiles are referred to as herbivory-induced plant volatiles (HIPVs) (Kessler & Baldwin, 2001) and oviposition-induced plant volatiles (OIPVs) (Hilker & Meiners, 2006; Hilker & Fatouros, 2015; Hilker et al., 2023). The induced volatiles attract enemies (predators, parasitoids) of the herbivore or its eggs. Both HIPVs and OIPVs can also affect defences in neighbouring plants and deter herbivore feeding and oviposition (War et al., 2012; Pashalidou et al., 2020). The emission of HIPVs varies depending on the herbivore’s feeding habits, with chewing insects generally inducing stronger responses than sap-feeders (Rowen & Kaplan, 2016; Davidson-Lowe & Ali, 2021). The emission of HIPVs is not just induced by the damage that a herbivore inflicts to a plant, but also by elicitors in herbivore oral secretions, leading to the release of various volatile compounds (Mostafa et al., 2022; Kutty & Mishra, 2023). The elicitors trigger complex signalling cascades involving membrane depolarization, calcium influx, and phytohormone modulation (Wu & Baldwin, 2009). The emission of OIPVs is induced by elicitors associated with the eggs; these elicitors are usually produced in the female’s reproduction tract and are released with the eggs to the plant surface where they stick the eggs to a leaf (Hilker & Fatouros, 2015; Hundacker et al., 2022).

While induced plant defences against ovipositing and feeding insects are well documented throughout the plant kingdom, most of our knowledge stems from studies on herbaceous plants, particularly angiosperms (Howe & Jander, 2008; Eyles et al., 2010). Much fewer studies addressed interactions between gymnosperms and folivorous insects. Most research on gymnosperm defence responses has focused on stem borers or feeders (Krokene, 2015). Consequently, our understanding of gymnosperm defences against leaf- or needle-chewing insects remains limited.

1.1.2 Inducible defence priming in plants

As constitutive defence of plants against herbivorous insects is resource-intensive and may come at a cost of growth and reproduction, inducible defence also has its disadvantages as it takes some time from perception of insect attack until effective mounting of defences (Zangerl, 2003; Backmann et al., 2019). To overcome this lag time an 'in between' option to constitutive and induced plant defence responses is the so called 'priming' of defence, which has first intensively been studied in plant - phytopathogen interactions (Conrath et al., 2002, 2006, 2015). The priming phenomenon depends on the perception of "warning cues" that predict future stress. These warning cues are termed priming stimuli; the warning cue may be a first mild stress, indicating an impending second, more severe stress, the so-called triggering stimulus (Hilker et al., 2016). But a warning cue may also be a *per se* stress-free environmental cue that reliably indicates future stress. For example, HIPVs released from damaged plants are known to be perceived by as yet undamaged, neighbouring plants as warning cues of herbivory; the warned plants start preparing their defences against the impending herbivory and are more effective than non-primed plants (Frost et al., 2008; Dicke & Baldwin, 2010; Karban et al., 2014; Erb et al., 2015). Primed plants respond faster or stronger or more sensitive to the triggering stimulus than non-primed plants (Hilker et al., 2016; Martinez-Medina et al., 2016). Exposure to a priming stimulus alone may have neutral or even negative effects on plant fitness in the absence of a subsequent triggering event. However, when the priming stimulus precedes a more severe triggering event, it is expected to confer fitness benefits to the plant (Hilker et al., 2016).

Anti-herbivore defence priming of plants has been reported in response to plant-derived cues like HIPVs and OIPVs, leaf damage, or broken trichomes (Peiffer et al., 2009; War et al., 2012; Pashalidou et al., 2020). In addition, volatile and non-volatile cues released from insects can prime plant anti-herbivore defences. These insect-derived priming cues include insect sex pheromones (Helms et al., 2013, 2017; Bittner et al., 2019) and contact cues such as insect footsteps on leaves (Hall et al., 2004; Hilker & Meiners, 2010; Hilker et al., 2016; Basu et al., 2018). Only a handful of studies have identified specific volatile cues perceived by plants as priming cues, and these have focused particularly on HIPVs (Engelberth et al., 2004; Runyon et al., 2006; Kost & Heil, 2006; Frost et al., 2008; Sugimoto et al., 2014; Erb et al., 2015).

Plant perception of volatiles released from insects, particularly pheromones, remains poorly understood so far.

1.1.3 Insect pheromones and plant responses

Chemical communication between plants and insects plays a crucial role in shaping ecological networks and plant-insect relationships (Beyaert & Hilker, 2014; Zu et al., 2023). Insects are known to produce highly specific chemical signals known as pheromones for social communication and organisation as well as for mate attraction (Ayasse et al., 2001; Yew & Chung, 2015).

On the other hand, these insect-derived volatiles seem to provide reliable chemical cues that plants could employ to defend themselves. Recent advancements have shed light on how the exposure of a plant to volatile insect pheromones affects plant defences against herbivorous insects. Helms et al. (2013) were the first to demonstrate that plants can perceive and respond to volatile compounds released by insects. For instance, *Solidago altissima* (goldenrod) plants exposed to volatiles from male goldenrod gallflies exhibited enhanced defence responses when subsequently infested by herbivores. The specific volatile compound identified was *E,S*-conophthorin, which increased the feeding-induced levels of jasmonic acid, a key hormone in plant defence. This effect was linked with reduced feeding damage from a specialised leaf beetle, *Eurosta solidaginis* (Helms et al., 2017). These findings suggested that insect pheromones can prime plants for improved defence, making them more resistant to attacks by herbivorous insects.

Similarly, Scots pine (*Pinus sylvestris* L.) exposed to sex pheromone components of the pine sawfly (*Diprion pini* (L.)) showed enhanced defence mechanisms against sawfly eggs. A study by Bittner et al. (2019) revealed that exposure to these pheromones led to a higher egg-induced accumulation of reactive oxygen species (ROS) in pine needles, which are crucial for plant defence. The ROS accumulation in pheromone-exposed, egg-laden pine needles was significantly higher than in unexposed, egg-laden ones. Survival rates of *D. pini* eggs were significantly reduced on previously pheromone-exposed Scots pine. This indicates that Scots pine can use insect pheromones as cues to enhance its defences against insect infestation.

The interaction between plant responses and insect pheromones is a fascinating example of co-evolution. This ability of plants to detect and respond to pheromones, thereby enhancing their defensive capabilities can in turn also lead to changes in behavioural responses of insects to the primed plants. For instance, goldenrod gallfly females were observed to avoid laying eggs on pheromone-exposed plants, likely due to the primed defence mechanisms in these plants (Helms et al., 2013, 2017).

1.2 General Overview of the Studied Species

1.2.1 *Pinus sylvestris* and *Diprion pini*

Pines (*Pinus* spp.) are evergreen gymnosperms and constitute the largest genus of the conifer family Pinaceae, encompassing over 100 species (Keeley, 2012). They are predominantly distributed across temperate regions in the northern hemisphere (Nobis et al., 2012). Pine trees play crucial ecological roles in various ecosystems worldwide, especially in temperate regions of the northern hemisphere, where they dominate landscapes and contribute significantly to ecological stability. They serve as carbon sinks, mitigating greenhouse effects through extensive

biomass accumulation (Rodrigues-Corrêa et al., 2012), and provide valuable resources like timber and resin (Oyen, 2006; Rodrigues-Corrêa et al., 2012).

A popular representative of pine species is *P. sylvestris*, commonly known as Scots pine. The species is both drought-resistant and frost-hardy, with a long lifespan. This gymnosperm is also the most distributed *Pinus* genus in the world and the second most distributed conifer tree in the northern hemisphere (Carlisle & Brown, 1968; Durrant et al., 2016). It grows in central and eastern Europe, throughout nearly all parts of Scandinavia, and is also found in Russia and various parts of Asia, notably Siberia. Scots pine is systematically categorised within the conifer lineage of gymnosperms, alongside three other extant lineages: cycads, gnetophytes, and Gingko (Mathews, 2009).

Several herbivorous insect species are specialised to feed on Scots pine as their host plant (Mumm & Hilker, 2006). Pine trees are renowned for their extensive production of volatile and non-volatile terpenes (Kopaczyk et al., 2020), which serve as cues for specialised herbivores to locate hosts and can also function as defensive mechanisms (Mumm & Hilker, 2006).

The conifer sawflies (Hymenoptera: Diprionidae) are a small family of folivorous insects primarily associated with Pinaceae (Codella & Raffa, 2002). Diprionidae are holarctic, with extensions into northern Central America, Cuba, Thailand, northern India, and northern Africa (Smith, 1993). They are among the most harmful defoliators of pines (*Pinus* spp.) in the northern hemisphere (Averill et al., 2017; Davis et al., 2023). Several destructive sawfly species, such as *Diprion similis* (Hartig) and *Neodiprion sertifer* (Geoffroy), were introduced into North America from Europe, entering a region with abundant food and lacking their native parasitoid-predator complex (Smith, 1993). Economic losses due to reduced growth and tree mortality caused by sawfly infestation can be substantial, with estimates ranging from $40 to $310 per hectare (Lyytikäinen-Saarenmaa & Tomppo, 2002). The impact varies based on defoliation intensity, outbreak duration, and species characteristics, with gregarious species showing higher outbreak frequencies (Hanski, 1987). Several genera of diprionid sawflies are known in Europe, including *Diprion, Gilpinia, Neodiprion,* and *Monoctenus* (Benson, 1939; Smith, 1974). These genera can be distinguished by characteristics such as wing venation, cenchri size, and mesoscutellum shape (Benson, 1939; Smith, 1974). *Diprion* and *Gilpinia* are particularly important in Europe, with species like *D. pini, D. similis,* and *G. polytoma* (formerly *D. polytomum*) causing significant defoliation of pine forests (Benson, 1939; Balch et al., 1941; Kangas, 1962).

The hymenopteran species *D. pini* is primarily associated with *P. sylvestris*, showing a strong preference for this host (Auger & Géri, 1993; Barre et al., 2002). This species is found throughout Europe, Asia (Russia and Turkey), and northern Africa. In Central Europe, *D. pini* is described as univoltine or bivoltine, depending on climatic conditions (Eichhorn, 1991). Sawfly adults are non-feeding and highly specialised for reproduction (Jewett et al., 1976). Females release sex pheromones, specifically (2*S*,3*R*,7*R*)-3,7-dimethyl-2-tridecanyl acetate and propionate, to attract males prior to mating (Bergström et al., 1995; Anderbrant et al., 2005). Females lay their eggs in rows within pine needles using their saw-like ovipositor, which slits the needle open to deposit several eggs into the longitudinally slit needle (Bittner et al., 2019; Hundacker et al., 2024). The oviposition of *D. pini* causes severe damage to the needle, thus differing from many other insect species that only slightly harm the plant or glue their eggs onto the plant surface (Meiners & Hilker, 2000). Sawfly larvae consume the needles of the host trees where the females

oviposited. Their feeding damage may result in reduced growth and sometimes reduced survival of the host trees (Bergström et al., 1995). Most sawflies, including *D. pini* and *D. similis*, are arrhenotokous, meaning unfertilised eggs develop into males, while mated females produce both male and female progeny (Coppel & Benjamin, 1965; Heimpel & de Boer, 2008).

While *D. pini* can survive on other *Pinus* species, its performance and development are generally inferior compared to *P. sylvestris* (Auger & Géri, 1993; Barre et al., 2002). The insect's host selection is influenced by various factors, including foliage characteristics like chemical composition (Pasquier-Barre et al., 2001; Barre et al., 2003). The oviposition preference and larval survival of *D. pini* vary among *P. sylvestris* clones (Pasquier-Barre et al., 2000). While *D. pini* primarily targets *P. sylvestris*, it may pose a potential threat to other pine species if these have been introduced to new environments (Auger & Géri, 1993; Łukowski et al., 2022).

1.2.2 Interactions between pine, pine sawflies and their antagonists

The tripartite interaction of *P. sylvestris, D. pini*, and the egg parasitoid *Closterocerus ruforum* (Krausse) shows complex relationships between different trophic levels. The host range of *C. ruforum* is limited to the eggs of species within the subfamily Diprioninae (Pschorn-Walcher & Eichhorn, 1973; Eichhorn & Pschorn-Walcher, 1976), which primarily feed on pines (*Pinus spp.*) in Europe (Pschorn Walcher, 1982). Based on the definition by Steidle & Loon (2003), the egg parasitoid *C. ruforum* is thus classified as a specialist at both the host and host plant level.

Current knowledge about non-primed, indirect defence of pine against sawfly eggs involving the attraction of *C. ruforum* can be summarised as follows: For effective recruitment of parasitoids by the plant, *P. sylvestris* is expected to respond to the eggs in a timely manner. Egg deposition by *D. pini* is known to induce the release of volatiles (OIPVs) attractive to *C. ruforum*. Three days post egg deposition by *D. pini*, the egg-induced odour of pine needles attracts the egg parasitoid *C. ruforum*, which kills the sawfly eggs (Hilker et al., 2002; Hilker & Meiners, 2002). Only those parasitoid females are attracted, which have previously experienced (odour of) egg-laden pine; naïve ones are not attracted (Schröder et al., 2008). The attractive odour is characterised by higher quantities of the sesquiterpene (*E*)-beta-farnesene (EBF) when compared to egg free pine (Mumm et al., 2003). Electroantennogram (EAG) studies revealed that the egg parasitoid's antennae are physiologically responsive to EBF, but also to some other terpenes. Behavioural assays showed that the egg parasitoid needs enhanced quantities of EBF mixed with four other EAG-responsive pine terpenes in ratios as they occur in egg-laden pine to be attracted. When mixing EBF with the respective four other EAG-responsive terpenes in ratios as EBF and the four other terpenes occur in odour of egg-free pine, no attraction is detectable (Beyaert et al., 2010).

The parasitoid *C. ruforum* is also attracted to *D. pini* sex pheromonal components (Hilker et al., 2000), i.e. to (2*S*,3*R*,7*R*)-3,7-dimethyl-2-tridecanyl acetate and propionate. However, no residues of *D. pini* pheromone were detected on pine needles that had been exposed to the pheromones for 24 hours and then analysed a day later (Bittner et al., 2019, supplement). Therefore, the release of egg-induced pine odour three days past egg deposition (and probably several days after pheromone release and mating) might be considered a plant-derived back-up to the attractive effect of the sawfly’s sex pheromones.

Pine twigs treated with jasmonic acid also emit volatiles that attract egg parasitoids (Hilker et al., 2002). However, volatiles from pine twigs artificially wounded to mimic the mechanical damage by the sawfly ovipositor are not attractive. Applying the *D. pini* oviduct secretion, which coats the eggs, into wounded pine needles induces volatiles that attract egg parasitoids (Hilker et al., 2002). Hundacker et al. (2022) could recently isolate the elicitor of pine OIPVs from *D. pini* oviduct secretion and identified it as an annexin-like protein, named diprionin.

1.3 Gaps in Knowledge and Research Questions Addressed in this Thesis

Pine trees enhance their direct defences against sawfly eggs following exposure to the sex pheromones of the sawfly *D. pini;* the survival rate of sawfly eggs on previously pheromone-exposed pine trees was found to be significantly reduced (Bittner et al., 2019). Since diprionid sawflies have a long-standing co-evolution with their host plants (O'Reilly et al., 2015), **the question arises whether females avoid oviposition on previously pheromone-exposed pine,** thus protecting their eggs from increased mortality, which the eggs would suffer on pheromone-primed trees. If such avoidance of pheromone-exposed pine by egg-laying sawfly females can be observed, it could be due to either pheromone-induced, deterrent odours emitted by these trees or to pheromone-induced pine needle contact cues that affect *D. pini* females. Helms et al. (2013, 2017) showed that goldenrod plants are primed for improved defence against chrysomelid larvae by prior exposure to the volatiles released from male gallflies. When exposing goldenrod plants to the volatiles of young gallfly males, the gallfly females avoided oviposition on these plants, while they preferred oviposition on plants exposed to older males (Yip et al., 2020).

Additionally, while priming of pine direct defence against sawfly eggs by prior exposure to *D. pini* sex pheromones has been proven (Bittner et al., 2019), we do not know whether exposure of pine to sawfly sex pheromones also affects direct defence against hatching larvae. Current knowledge about priming of direct pine defence against sawfly larvae can be summarised as follows: Direct defence of pine against *D. pini* larvae is primable by prior sawfly egg deposition. We know that *D. pini* larvae feeding on previously egg-primed trees perform worse and that the surviving larvae develop into adult females with reduced fecundity (Beyaert et al., 2010). **Whether this egg-mediated priming effect against sawfly larvae can even be topped by prior exposure of pine to sex pheromones is an open question**.

Moreover, when considering the non-primed, indirect defence pine defence induced by sawfly eggs (as mentioned earlier, section 1.2.2), **the question comes up whether exposure of pine to *D. pini* sex pheromones enhances the indirect defence of pine trees by priming them to produce higher quantities of specific volatiles upon egg deposition**. This could improve the efficiency and effectiveness of recruiting parasitoids like *C. ruforum* to the sawfly eggs, thus increasing egg mortality and reducing the impact of sawfly infestations.

One remarkable characteristic of pine trees is their ability to retain their needles for longer periods than most deciduous trees (Armour et al., 2003). In evergreen conifers, needle age affects morphological and chemical traits. Younger needles typically have higher nitrogen but lower concentrations of defensive compounds like phenolics (Hatcher, 1990; Moreau et al., 2003; McCall & Fordyce, 2010). Limited knowledge exists regarding how conifer-feeding insects, particularly conifer sawflies (Hymenoptera: Diprionidae), respond to the varying qualities of

current-year versus previous-year needles (Schwenke, 1982; Davis et al., 2023). Several studies have examined the effects of needle traits on sawfly performance (Niemala et al., 1982; Larsson et al., 1986; Björkman & Gref, 1993; Lyytikäinen, 1994; Pasquier-Barre et al., 2001; Moreau et al., 2003; Giertych et al., 2007). However, pine needle phytohormone concentrations related to defences against folivorous insects remained uninvestigated prior to this thesis, as did the roles of proteinase inhibitors (PIs), which are known in angiosperms to vary with plant age and to impact protein digestibility in insects (Wolfson & Murdock, 1990; Van Dam et al., 2001; Zhu-Salzman & Zeng, 2015). Therefore, **the question comes up whether phytohormone concentrations and PI activities of pine needles also show age-dependent changes in response to herbivory, as known for angiosperm foliage.** In addition**, it is an open question whether *D. pini* shows different behavioural responses to young and old (previous-year) pine needles.**

In summary, in this thesis I addressed the above-mentioned gaps in knowledge by exploring a series of targeted research questions, each aligning with a specific chapter. An overview of the questions addressed is visualized in Figure 1.

Chapter 2: Pine Response to Sawfly Pheromones: Effects on Sawfly's Oviposition and Larval Growth

(i) Do female *D. pini* sawflies avoid ovipositing on pine trees exposed to *D. pini* sex pheromones, possibly as a strategy to protect their eggs from enhanced mortality on pheromone-primed trees?
(ii) Does exposure of pine to *D. pini* sex pheromones enhance the direct defences of egg-free pine trees against feeding damage by sawfly larvae?
(iii) How does the combination of (i) exposure to *D. pini* sex pheromones plus (ii) the egg-mediated priming effect of pine defence against larvae affect the survival and development of *D. pini* larvae?

Chapter 3: Sawfly Sex Pheromones: Analysis of Their Impact on Pine Odor Attractive to Egg Parasitoids

(i) Can exposure of pine to *D. pini* sex pheromones enhance the indirect defence mechanisms of pine trees, specifically by increasing the release of volatiles that attract egg parasitoids like *C. ruforum*?

Chapter 4: Needle Age-Dependent Defence of Scots Pine Against Insect Herbivory

(i) How does needle age influence the oviposition and larval feeding preferences of *D. pini*, and what impact does needle age have on the survival and performance of *D. pini* eggs and larvae?
(ii) How do the phytohormonal concentrations differ between control pine needles and feeding-damaged needles of different age?
(iii) What is the proteinase inhibitor activity in control and feeding-damaged needles of different age?

By addressing these research questions (Chapters 2 to 4), I provide novel insights into how exposure of pine to sawfly sex pheromones as well as pine needle age affect pine defences. The **general discussion** (**Chapter 5**) of this thesis then integrates these findings within a broader

context of current research alongside highlighting the implications of these novel findings for future studies and offering insights that could drive further advancements in this field.

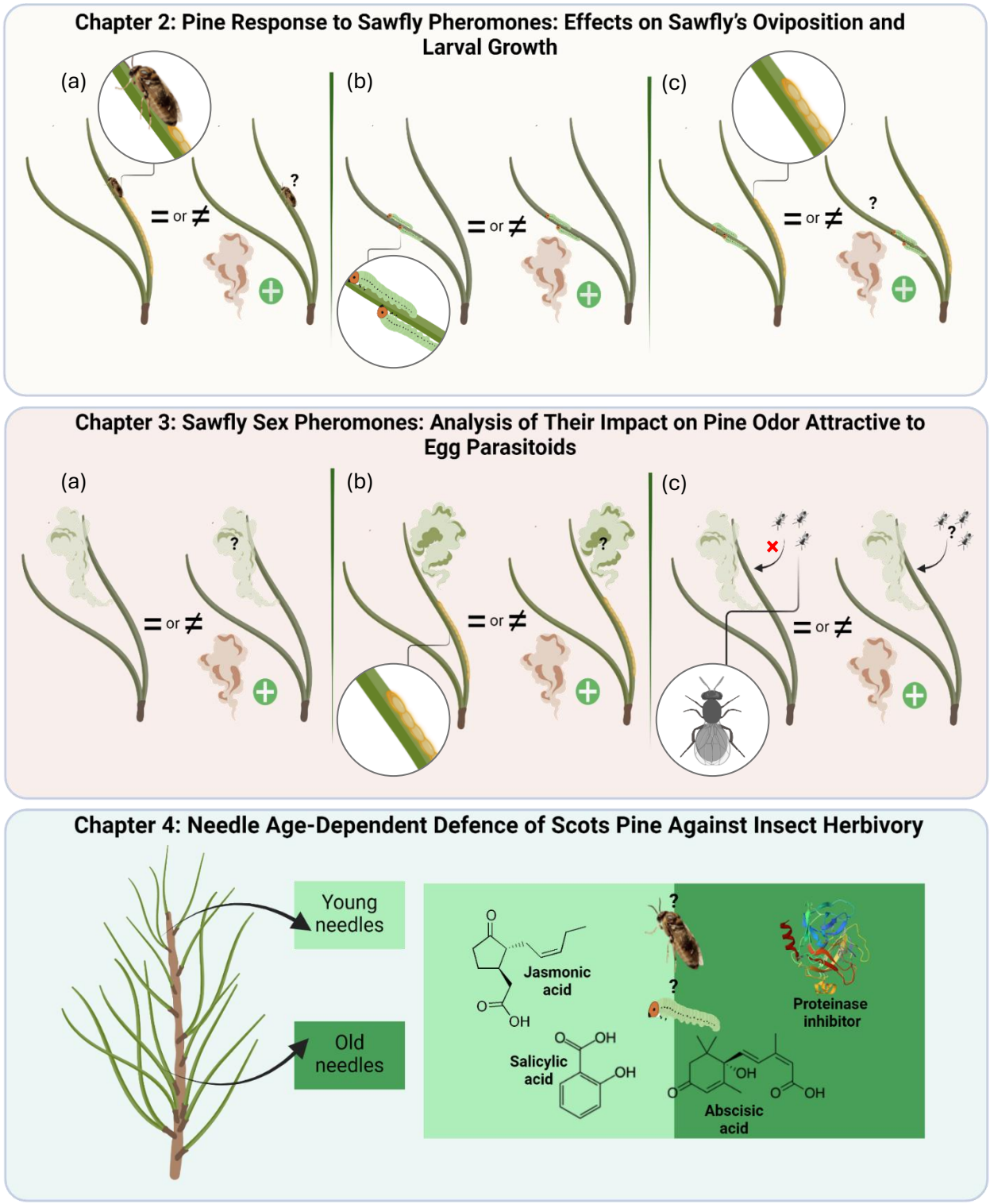

Figure 1: Schematic visualisation of the main research questions addressed in this thesis. Chapter 2 (top frame) investigates whether exposure of *Pinus sylvestris* to *Diprion pini* sex pheromones influences (a) sawfly oviposition (or not) and (b) the performance of larvae feeding on egg-free pine (or not), and (c) the performance of larvae feeding on previously egg-laden pine (or not). Chapter 3 (middle frame) examines whether exposure of (a) egg-free pine to *D. pini* sex pheromones affects pine volatile emissions (or not), (b) egg-laden pine to *D. pini* sex pheromones affects pine volatile emissions (or not), and (c) egg-free pine to *D. pini* sex pheromones attracts the egg parasitoid *Closterocerus*

ruforum (or not). Chapter 4 (bottom frame) explores how needle age affects sawfly oviposition, larval feeding preferences, and defence-related changes of phytohormone concentrations and proteinase inhibitor activity in pine needles. © 2025, Asifur Rahman Soad. Created with BioRender.com.

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Chapter 2: Pine Response to Sawfly Pheromones: Effects on Sawfly's Oviposition and Larval Growth

insects

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Pine Response to Sawfly Pheromones: Effects on Sawfly's Oviposition and Larval Growth

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Simple Summary: Mass outbreaks of the pine sawfly *Diprion pini* can cause severe damage to pine forests. The larvae of this herbivorous insect feed selectively on the needles of pine trees, notably *Pinus sylvestris.* During mass outbreak periods, the females release large amounts of sex pheromones. A prior study revealed that the survival rate of sawfly eggs laid on pheromone-exposed pine needles was lower than that of eggs on unexposed pine. In our study, we found that *D. pini* females avoided oviposition on pheromone-exposed pine, possibly as a counter-adaptation to the enhanced defenses of previously pheromone-exposed trees against sawfly eggs. The females only discriminated between pheromone-exposed and unexposed trees when they had the chance to touch the needles, but not when exposed to the odor of these types of trees. However, the performance of larvae did not significantly differ on pheromone-exposed and unexposed trees. These results underscore the complexity of the chemical ecology of sawfly–pine interactions and highlight the nuanced roles that pheromones play in shaping the relationships between herbivores and their host plants.

Abstract: Insect pheromones have been intensively studied with respect to their role in insect communication. However, scarce knowledge is available on the impact of pheromones on plant responses, and how these in turn affect herbivorous insects. A previous study showed that exposure of pine (*Pinus sylvestris*) to the sex pheromones of the pine sawfly *Diprion pini* results in enhanced defenses against the eggs of this sawfly; the egg survival rate on pheromone-exposed pine needles was lower than that on unexposed pine. The long-lasting common evolutionary history of *D. pini* and *P. sylvestris* suggests that *D. pini* has developed counter-adaptations to these pine responses. Here, we investigated by behavioral assays how *D. pini* copes with the defenses of pheromone-exposed pine. The sawfly females did not discriminate between the odor of pheromone-exposed and unexposed pine. However, when they had the chance to contact the trees, more unexposed than pheromone-exposed trees received eggs. The exposure of pine to the pheromones did not affect the performance of larvae and their pupation success. Our findings indicate that the effects that responses of pine to *D. pini* sex pheromones exert on the sawfly eggs and sawfly oviposition behavior do not extend to effects on the larvae.

Keywords: pine; sawfly; herbivore; insect egg; sex pheromone; oviposition; behavior; olfaction; co-evolution

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1. Introduction

Chemical communication via pheromones plays an important role in the insect world. Insect pheromones convey complex information between conspecific individuals about, e.g., the presence of conspecifics, mating partners, territorial boundaries, paths to resources, or danger [1–4]. Insect pheromones, which are perceived by olfactory receptors, are

volatile compounds of various chemical structures [5,6]. While a plethora of studies have addressed the ecology, physiology, and molecular basis of insect communication via volatile pheromones [2,7–9], scarce knowledge is available on how these chemicals affect plant responses, which in turn may shape the insect's behavior, development, or survival.

Recent advancements have shed light on how the exposure of a plant to volatile insect pheromones affects that plant's defenses against herbivorous insects. Helms et al. [10,11] were the first to show that plant perception of an insect pheromone results in improved plant defense responses to subsequent insect infestation. In general, cues that "warn" a plant of impending stress and result in subsequent, improved stress responses are referred to as "priming" stimuli [12,13]. Defense priming is a process which prepares the plant to respond more rapidly or more intensively to future attack [13–15]. In addition to insect pheromones, several other cues are known to prime plant anti-herbivore defenses, among them being herbivory-induced and oviposition-induced plant volatiles (HIPVs and OIPVs), insect eggs, and vibrations caused by leaf-feeding larvae [16–19]. The research by Helms et al. [10] on goldenrod plants (*Solidago altissima*) revealed that exposure of the plants to volatiles released from male goldenrod gall flies, *Eurosta solidaginis*, elicited enhanced the anti-herbivore defense responses of the plant, resulting in reduced feeding damage by a leaf beetle specialized upon goldenrod plants. Pheromone-exposed, feeding-damaged goldenrod plants showed higher levels of jasmonic acid than unexposed, feeding-damaged plants. The volatile compound released by male *E. solidaginis* that evokes the enhanced feeding-induced defense in goldenrod plants was identified as *E*,*S*-conophthorin, a spiroacetal [11]. Interestingly, goldenrod gallfly females avoided egg deposition onto previously pheromone-exposed plants.

Similarly, exposure of Scots pine (*Pinus sylvestris*) to the sex pheromone components of the herbivorous pine sawfly *Diprion pini* were shown to affect the tree's defense responses [20]. Pine trees exposed to these pheromones showed improved defense against eggs of this sawfly. Fewer eggs survived on trees exposed to the pheromonal components (2*S*,3*R*,7*R*)-3,7-dimethyl-2-tridecanyl acetate and propionate. These compounds can attract *D. pini* males over great distances [21,22], indicating that the pheromone can be widely transferred in a pine forest. Sawfly egg deposition induces accumulation of H_2O_2 in pine needles. The pheromone-exposed trees with sawfly eggs accumulated more H_2O_2 than unexposed trees with eggs, suggesting that ROS (reactive oxygen species) signaling is involved in this pheromone-mediated, improved plant defense against insect eggs [20].

Here, we addressed the question of whether *D. pini*, highly specialized on pine, evolved an oviposition behavior that allows it to cope with the host plant's primability of defenses against the eggs. A strategy to enhance the survival rate of eggs would be avoidance of areas with high pheromone concentrations. However, a previous study showed that *D. pini* females do not physiologically respond to their own pheromones [20]. Thus, they cannot use them as an abundance sensor, as some lepidopteran species can do [23]. We tested whether *D. pini* females avoid oviposition on pheromone-exposed pine. Such an avoidance behavior might be due to deterring pheromone-induced pine volatiles or contact cues. Furthermore, we investigated whether the detrimental effect that pheromone-exposed pine exerts on the egg survival rate extends to detrimental effects on larvae that hatch from the surviving eggs.

In detail, we addressed the following questions:

Can females of *D. pini* discriminate between odor released from pheromone-exposed trees and odor from untreated trees?

Do females of *D. pini* oviposit as readily on pheromone-exposed trees as on untreated trees?

Does exposure of pine to the pheromones affect larval performance and pupation rate?

Answers to these questions may shed light on the pine–pine sawfly interactions, which developed over a long evolutionary time [24] and resulted in adaptations and counter-adaptations of both players, thus forming robust host plant–herbivore interactions.

2. Materials and Methods

2.1. Insect and Plant

Diprion pini were reared in a climate chamber under conditions of 20 °C, 18 h:6 h light:dark, and 70% relative humidity. Scots pine twigs were collected from the Grunewald Forest in the south-west of Berlin, placed in vases filled with tap water, and enclosed in a transparent 14.9 L Plexiglas® cylinder (height: 50 cm, diameter: 19.5 cm), which was closed with a gauze lid on top. Adult *D. pini* were introduced into the setup for mating and egg deposition. Usually, 12 to 14 days after egg deposition, larvae began to hatch and started feeding on the needles. During larval development, larvae were provided with Scots pine twigs ad libitum. The larvae show five (male) to six (female) instars until pupation [25]. Upon the onset of pupation, the cocoons were collected and stored in a refrigerator at 4 °C in the dark.

For the experiments, three-year-old *P. sylvestris* trees (40 to 60 cm in height with a stem diameter ranging between 2 and 4 mm) were acquired from a local tree nursery (Baumschule Stackelitz GmbH & Co. KG; 06,868 Coswig/OT Stackelitz; Germany). These small trees were grown individually in pots filled with Classic T potting soil (Einheitserde®, a mixture of peat and clay; N = 340 mg·L^{-1}, P_2O_5 = 260 mg·L^{-1}, K_2O = 330 mg·L^{-1}). The trees were initially kept in a greenhouse under long-day conditions (20 °C, 18 h:6 h light:dark) until the start of the experiments. One week prior to the start of the experiments, the trees were transferred to a climate chamber for acclimation to the abiotic experimental conditions (20 °C, 18 h:6 h light:dark, 70% relative humidity). Each potted tree was enclosed in a transparent 14.1 L Plexiglas® cylinder (height: 80 cm, diameter: 15 cm) with an air inlet at the bottom and an air outlet on top. Each cylinder was ventilated by charcoal-filtered air at a rate of 250 mL·min^{-1} through an inlet at the bottom; the air flowrate on top was the same, thus ensuring that only charcoal-filtered air was inside the cylinder. To prevent volatile organic compounds (VOCs) released from the soil or the roots from affecting aboveground pine responses, the pots were wrapped with polyethylene terephthalate (PET) bags covering the pots and soil.

Pine trees were exposed to the synthetic male-attracting sex pheromones of *D. pini* females, specifically (2*S*,3*R*,7*R*)-3,7-dimethyl-2-tridecanyl acetate and propionate, for treatments (supplied by the laboratory of Olle Anderbrant, Lund University, Sweden). The pheromone esters were dissolved in hexane, each at a concentration of 50 ng·µL^{-1}.

For pine treatments with pheromones, 100 µL pheromone solution dissolved in hexane (50 µL acetate + 50 µL propionate) was applied to a cotton wool pad (5.6 cm diameter and 0.4 cm thickness) as a dispenser. Control trees were supplied with a solvent control (100 µL hexane on a cotton wool pad). To allow solvent evaporation, the cotton pads were kept under a fume hood for 30 min before exposing them to the trees. After this time, the highly volatile hexane was expected to have evaporated, and a pad with pheromones was placed into the aforementioned 14.1 L Plexiglas® cylinder with a pine tree for 24 h. Additionally, for a second control (blank), an untreated cotton pad without any solvent was added to a cylinder with a pine tree. After a treatment period of 24 h, the pads were removed from the cylinders. The chosen amount of pheromones estimates an equivalent of 270 to 450 females per tree, which comes close to the actual numbers in a mass outbreak [20].

For the larval performance assays (compare Section 2.4), in addition to pheromone-treated, hexane-treated, and untreated trees, we also used trees that were exposed to the pheromone components and afterwards subjected to egg deposition by two *D. pini* females for a duration of three days; the females had the chance to mate with two *D. pini* males during this time. Thus, we could study larval performance on pheromone-treated and subsequently egg-laden pine. For a control, hexane-treated trees as well as "blank" trees were exposed to *D. pini* egg deposition and subsequently to the larval performance assay.

2.2. Olfactory Choice Assay

To test how *D. pini* females responded to the odor of pheromone-exposed trees, we conducted a choice experiment as described by Hilker & Weitzel [26]. Female *D. pini* were

allowed to crawl upwards along a Y-shaped glass rod (length of base stem: 35 cm, length of diverging Y-legs: 20 cm; diameter of the stem and legs: 1 cm; distance and angle between Y-legs on top: 60 cm; 90°). The setup was illuminated with a light positioned 80 cm above the base of the Y-glass rod (Philips TL-E 29-530 bulb, 22 Watt), which encouraged the females to walk upwards readily. The bioassay was carried out at 20 °C room temperature.

An untreated control tree was placed at the end of one of the Y-legs and a pheromone-exposed tree at the end of the other Y-leg. While crawling upwards along the glass rod, females could not touch the plants. When a female reached the end of one of the legs, the choice was recorded. The maximum observation period per female was 5 min. Females that did not choose one of the Y-legs within this observation period were considered to have made no choice.

We tested plants one day, two days, and five days after treatment. For each time, three plant pairs were tested, with 11 to 13 females per plant pair. After having tested four to five females, the rod was turned by 180°. For each plant pair, a new clean glass rod was used.

2.3. Oviposition Assay

To evaluate the oviposition behavior of *D. pini* in response to pine trees treated with pheromones, as well as those treated with hexane or left untreated, a no-choice experiment was performed. In the end of the 24 h-treatment period of a pine tree (compare Section 2.1), two *D. pini* females and two males were introduced into a Plexiglas® cylinder (Nordic Panel GmbH, Stade, Germany) containing a tree. Females and males were used that had emerged two to three days before from the cocoons and had no chance to mate prior to the assay. These insects were observed over a period of five days to assess their egg-laying activity. The abiotic conditions were 20 °C, 70% relative humidity, and 18 h:6 h light:dark. The experiment was repeated five times, with each round including an equal number of trees per tested group (n = 22 trees per group in total). After the five-day period, we recorded the number of trees with and without eggs, as well as the number of eggs on egg-laden trees.

2.4. Larval Performance Study

To investigate the effect of sawfly sex pheromones on pine defenses against larvae, we allowed larvae to feed on trees exposed to the pheromones, to the solvent hexane, or left untreated. Five larvae (two to three days old) were taken from the rearing setup and placed on each tree. Their weight was recorded after 3, 9, and 15 days of feeding. Post weighing them after a 15-day feeding period, larvae were transferred to new untreated trees to monitor their development until pupation. A transfer to new trees was necessary because the larvae had eaten up almost all needles from the trees onto which they initially had been placed. Transfer of larvae to new, untreated trees allowed the determination of how a 15-day feeding period on pheromone-trees affects pupation success.

A second larval performance study was conducted to explore if pine exposure to sawfly pheromones enhances egg-mediated anti-herbivore defenses. Therefore, *D. pini* larvae were allowed to feed on trees previously exposed to pheromones (or to hexane or left untreated) and subsequently treated with *D. pini* egg deposition (compare Section 2.1). Thirteen days post egg deposition, five *D. pini* larvae (two to three days old) were taken from the rearing and introduced to these trees. This timing aligns with the natural egg incubation period under the given abiotic conditions (20 °C, 70% relative humidity, and 18 h:6 h light:dark); in nature, hatching larvae start feeding at their natal site. To maintain a controlled number of five larvae feeding on the trees, we killed the eggs on the trees by piercing them with sterile needles shortly before the larvae could hatch.

Both larval performance studies were conducted in multiple repeats. Each repetition included trees from all different treatments, with five larvae per tree. Larvae were individually weighed, serving as biological replicates. Variability in biological replicates was due to larval mortality and recovery issues throughout the experiments.

2.5. Statistics

Graphing and data analysis were conducted using GraphPad Prism (V 10.2.1) (GraphPad Software, Boston, MA, USA). Initially, the data underwent a normality test using the Shapiro–Wilk method, followed by appropriate statistical tests.

The non-normally distributed data of the olfactory dual choice assay were evaluated by the Wilcoxon matched-pairs signed rank test. Since the data of the oviposition assays and the larval performance study were also non-normally distributed, we compared the data obtained with pheromone-exposed, hexane-exposed, and untreated pine with Kruskal–Wallis tests. In cases of significant treatment effects, a Kruskal–Wallis test was followed by Dunn's tests for dual comparisons. Pairwise comparisons of the weight of larvae on egg-free and egg-laden pine trees (previously treated, or hexane-exposed, or pheromone-exposed) and of the larval pupation rate on these types of trees were conducted by Mann–Whitney *U* tests. *p*-values exceeding 0.05 were deemed not significant (ns), whereas *p*-values below 0.05 were classified as significant.

3. Results

3.1. Females of *D. pini* Do Not Discriminate between Odor from Pheromone-Exposed and Unexposed Pine

The olfactory choice assay revealed that the number of *D. pini* females that moved towards the odor released from pheromone-exposed *P. sylvestris* trees did not differ from the number of females that orientated towards the odor from unexposed trees. Regardless of the time past pheromone exposure (one, two, or five days), the results show no specific preference of the females for either the pheromone-exposed or control trees. A few females did not choose between the two odor sources within the observation period; they neither walked up the olfactometer Y-leg equipped with the pheromone-exposed tree nor the Y-leg with the control tree. They account for only 12–25% of all tested females when considering the three time intervals past pheromone exposure (Figure 1).

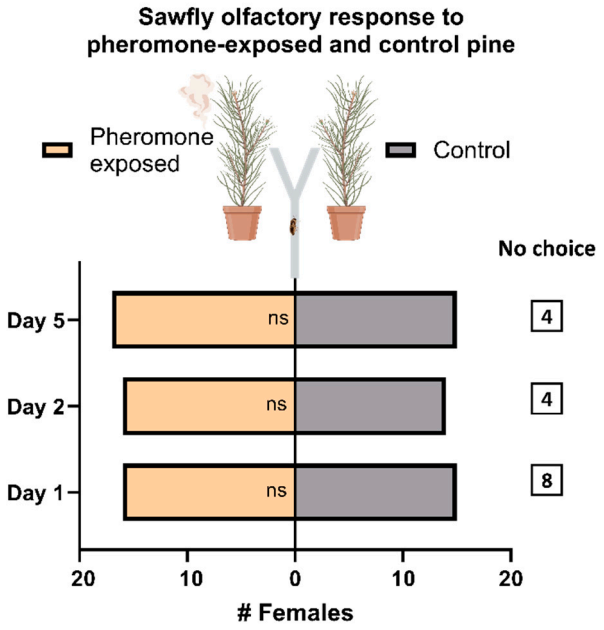

Figure 1. Responses of *Diprion pini* females to odor of pheromone-exposed and unexposed (control) *Pinus sylvestris* trees. Bioassay setup: Y-shaped glass rod: the end of one arm was equipped with a pheromone-exposed tree and the other arm with an untreated control tree. Female insects ascended the glass rod and selected one of the two Y-arms. The choice was evaluated at three time intervals: one, two, and five days after a 24 h-long pheromone exposure. Test period: five minutes. "No choice": number

of females that did not select either arm within the test period. Each test day included three pairs of plants, each with n = 11–13 *D. pini* females, resulting in a total of n = 34–39 females for each test day. Wilcoxon matched-pairs signed rank test; no significant ($p > 0.05$) differences between the number of females responding to the odor of the control tree and the pheromone-exposed tree (indicated by "ns").

3.2. Ovipositing D. pini Females Prefer Trees without Prior Pheromone Exposure, but If Chosen, the Number of Eggs on Pheromone-Exposed Trees Does Not Differ from That on Unexposed Trees

When counting the number of trees that received *D. pini* eggs, the type of pine treatment significantly affected which tree was chosen for egg deposition (Figure 2a). Significant treatment effects (p = 0.0137) were detected by a Kruskal–Wallis test. Notably, a post hoc (Dunn's) test revealed no significant difference between the number of untreated and hexane-treated trees with eggs ($p > 0.9999$), indicating that the solvent used to dissolve the pheromone had no additional effect. However, *D. pini* females were by trend less likely to oviposit on pheromone-exposed trees than on untreated trees (p = 0.0561). Furthermore, significantly fewer pheromone-exposed trees than hexane-treated trees received egg depositions (p = 0.0216). These data indicate that *D. pini* females preferably lay their eggs on trees that have not been exposed to pheromones.

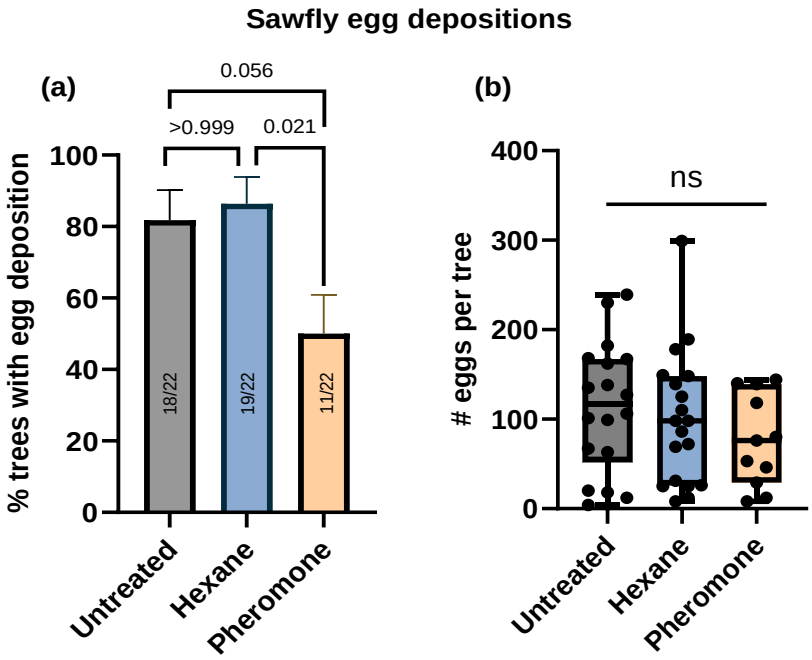

Figure 2. *Diprion pini* egg depositions. No-choice assay: an untreated, a hexane-exposed, or a pheromone-exposed *Pinus sylvestris* tree was offered separately to each two females and two males for five days. In total, n = 22 trees of each type were offered. (**a**) Mean percentage (±SE) of trees that received *D. pini* egg depositions and (**b**) number of eggs per tree that received egg deposition presented by box plots with medians, first and third quartiles, and full range of scores. (**a**,**b**) Kruskal–Wallis test followed by Dunn's multiple comparisons; in (**a**), exact p-values are given; in (**b**), ns not significant ($p \leq 0.05$).

When counting the number of eggs on trees that had been chosen by *D. pini* for egg deposition, no significant treatment effects were detected (p = 0.4705, Kruskal–Wallis test) (Figure 2b). These results suggest that while exposure of pine trees affects the initial selection of oviposition sites, the pheromone exposure does not significantly impact the overall number of eggs laid per tree (Figure 2b).

3.3. Performance of D. pini Larvae Is Not Affected by Exposure of Pine Trees to the Sawfly's Pheromones, but by Pine Responses to Sawfly Egg Deposition

Larvae of *D. pini* performed equally well on pheromone-exposed, hexane-exposed, and untreated trees. Their weight gain on these types of trees did not significantly differ. No effects of the pheromone or hexane exposure were detected, regardless of whether the trees had received *D. pini* eggs or not after the pre-treatment with pheromones or hexane (Figure 3).

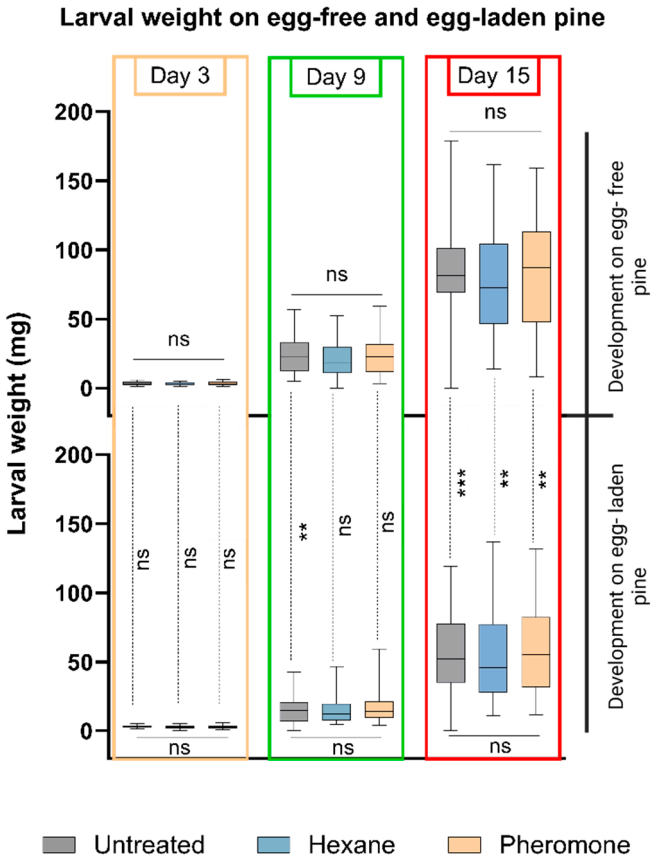

Figure 3. Weight of *Diprion pini* larvae on differently treated *Pinus sylvestris* trees. Types of trees offered without eggs (top) and with eggs (bottom): untreated, hexane-treated, and pheromone-exposed. Each treatment consisted of 7–10 trees and 5 larvae per tree. Young larvae were placed on a tree, and their weight was recorded after 3, 9, and 15 days of feeding. Bars show medians, first and third quartiles, and full range of larval weights. Within-day comparisons: Kruskal–Wallis test; ns not significant ($p > 0.05$). For comparison of larval weights on egg-free (top) and egg-laden trees (bottom): Mann–Whitney *U* test; significances indicated along vertical, dotted lines: ns not significant ($p > 0.05$), ** ($p < 0.01$), *** ($p < 0.001$).

However, when comparing the weight of larvae on trees with and without *D. pini* egg deposition, significant differences were detected. After a 15-day feeding period, larvae had gained more weight on egg-free pine trees than on previously egg-laden pine, regardless of the type of tree pre-treatment. When comparing the weight of larvae on previously untreated egg-free and egg-laden pine trees, a significant difference was detectable after a nine-day and fifteen-day feeding period (Figure 3). These findings suggests that pine responses to *D. pini* egg deposition enhance the tree's defense against *D. pini* larvae, consistent with the observations by Beyaert et al. [27]. Our study here indicates that this egg-mediated defense against *D. pini* is independent of prior exposure to sawfly sex pheromones.

Furthermore, when extending observations to track larval development up to pupation, we found that the number of larvae that successfully pupated did not significantly

differ between larvae on pheromone-exposed, hexane-exposed, and untreated trees, regardless of whether the trees had received eggs or not after the exposure to pheromones or hexane (Figure 4, Supplemental Table S1). Kruskal–Wallis tests revealed no significant treatment effects, neither within the egg-free group ($p = 0.8986$) nor within the egg-laden group ($p = 0.4416$). When comparing the numbers of successfully pupated larvae across all the tested conditions, no significant differences in pupation success were detected by a Kruskal–Wallis test ($p = 0.287$).

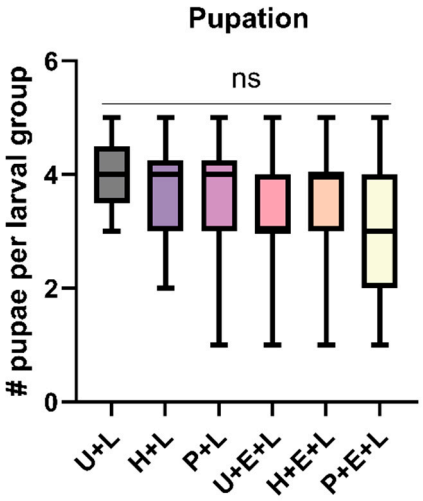

Figure 4. Number of pupae that resulted from a group of five *Diprion pini* larvae per tree. *Pinus sylvestris* trees with larvae were kept either untreated (U + L), hexane-exposed (H + L), or pheromone-exposed (P + L) and had no prior egg deposition; or the trees had received sawfly egg deposition prior to larval feeding and were left otherwise untreated (U + E + L), hexane-exposed (H + E + L), or pheromone-exposed (P + E + L) trees; n = 7–10 trees/treatment; 5 larvae/tree. Box plots with medians, first and third quartiles, and full range of scores are shown for each group. Kruskal–Wallis test; ns not significant ($p > 0.05$).

4. Discussion

Based on a previous study, which demonstrated that exposure of *P. sylvestris* to *D. pini* sex pheromones results in reduced egg survival rates, we investigated here whether *D. pini* females show a counter-adaptation to this egg-harming pine response to the pheromones. The results presented here provide evidence that sawfly females prefer egg deposition on pine that has not been exposed to these pheromones. We further addressed the question of whether the egg-harming response of pine to sawfly pheromones also affects larval performance. However, larval performance on pheromone-exposed pine did not significantly differ from the performance of larvae on trees that had not been exposed to the pheromones.

4.1. No Olfactory Discrimination by D. pini between Pheromone-Exposed and Unexposed Pine

The finding that the sawfly females did not discriminate between the odors of pheromone-exposed and untreated trees (Figure 1) might be due (i) to a lack of differences between the odor of untreated and pheromone-exposed pine, (ii) to the inability of *D. pini* females to discriminate between these odors, or (iii) to the type of olfactometer bioassay setup.

Ad (i): When considering the chemical nature of the *D. pini* pheromonal ester components, it is tempting to suggest that exposure of pine to them induces the release of pine volatiles. Plant responses to other carboxylic acid esters such as methyl salicylate, methyl jasmonate, or (*Z*)-3-hexenyl acetate have been shown to induce or prime anti-herbivore plant defenses [28–31], including those which rely on enhanced emission of plant VOCs, e.g., [28]. Whether exposure of pine to *D. pini* pheromones results in enhanced emission of terpenoid VOCs needs to be investigated by future chemical analyses comparing the volatile patterns of pheromone-exposed and untreated pine.

Ad (ii): Previous studies revealed that *D. pini* females are able to perceive pine volatiles (as might be expected from host plant volatiles) and to respond to them behaviorally, probably in a pine terpenoid-concentration dependent manner. High concentrations of pine terpenoid volatiles were shown to repel *D. pini* females [26]. However, this perception ability does not necessarily include the ability to detect possible differences in the odor of pheromone-exposed and untreated pine. Future studies need to elucidate the sawfly's olfactory abilities to discriminate between different pine volatile blends.

Ad (iii): In our olfactometer bioassay, we tested whether *D. pini* females preferred the odor released by untreated or by pheromone-exposed pine from a distance of about 20 cm. We cannot exclude that possible preferences for one of these odor sources become evident only at a shorter range. For example, in *Drosophila sechellia*, different olfactory receptors are relevant in long- and short-range attraction to an odor source [32].

4.2. Oviposition Preference of Unexposed Pine over Pheromone-Exposed Pine upon Contact

While *D. pini* females did not olfactorily discriminate between pheromone-exposed pine and unexposed pine, they discriminated between these types of trees when ovipositing. For the oviposition bioassay, the females were enclosed with either a pheromone-exposed tree or an unexposed tree, without the option to move to a different tree during this experiment. While the females enclosed with an untreated tree readily laid their eggs, the females enclosed with a pheromone-treated tree obviously were reluctant to lay their eggs, resulting in a lower number of pheromone-exposed trees than unexposed trees with eggs (Figure 2a). However, when the females decided to lay eggs on pheromone-exposed pine, the number of eggs laid on these trees did not differ from the number on unexposed trees (Figure 2b). This latter result might have been due to a high oviposition pressure of those females laying their eggs on the pheromone-exposed trees; in the conducted no-choice assay, they had no alternatives. For some plant–insect interactions, it is known that eggs that have been laid onto leaves induce a change in plant quality, which affects the plant's attractiveness for subsequent oviposition [33]. It is unknown so far how the changes that *D. pini* egg deposition induces in pine [20,25,34] affect the attractiveness of pine for subsequent sawfly egg deposition. Conifer sawflies are known to engage in a meticulous tactile examination of pine needles, involving walking up and down the needles and palpating them with their antennae, before egg laying [35]. Some species proceed to cut a 'test-slit' to further evaluate the needle quality [36,37]. Overall, our results suggest that the pheromone exposure changed tactile or close-range chemical cues in the final decision-making process for egg laying. While herbivorous insects often rely on plant volatiles when searching for host plants over long distances, contact cues may provide information about the suitability of the host [38–40]. Future studies should analyze whether pheromone-exposed pine needles exhibit changes in their cuticular chemical profile or patterns of volatiles relevant for close-range orientation of ovipositing *D. pini*.

Preference of unexposed over pheromone-exposed pine trees for egg deposition may be considered a counter-adaptation when taking into account that the egg survival rate on pheromone-exposed trees is significantly lower than on unexposed ones [20]. Exposure of goldenrod plants to the sex pheromone of male gallflies also reduced the number of gallfly ovipositions onto the pheromone-exposed plants when compared to unexposed plants [10]. However, whether exposure of goldenrod plants to gallfly pheromone results in reduced survival rates of gallfly eggs or reduced performance of gallfly larvae is unknown so far.

4.3. No Detrimental Effects of Pheromone-Exposed Pine on Larval Performance

While exposure of pine to *D. pini* sex pheromones affects sawfly oviposition behavior (Figure 2) and egg survival rate [20], it does not impact larval performance (Figure 3) and pupation success (Figure 4).

Numerous studies have investigated the relationship between insect oviposition preferences and offspring performance [41,42]. According to the preference-performance hypothesis (PPH) and "mother knows best" theory [43,44], females of herbivorous in-

sects are expected to lay their eggs preferentially onto plants where the offspring will develop and survive the best. Most of these studies focus on the relationship between oviposition preferences and larval performance [45–48]. However, in addition to the larval development and survival, the egg survival rate also needs consideration, especially when considering the multitude of defensive plant responses to insect egg deposition [18].

Therefore, we suggest that oviposition preferences might be much more tightly associated with egg survival rates than larval survival rates. Indeed, several studies did not find support for a tight link between oviposition preference and larval performance [41,47,48]. In specialized insects, this might be due to a wide range of factors, among them being, e.g., the availability of optimal host plants, the risk of predation, parasitization, or pathogen infection of the larvae, and a mismatch of the suitability of the host plants for the egg laying female and for the larvae [49]. However, these factors may also affect the relationship between oviposition preference and egg survival rates. Under laboratory conditions, exposure of pine to *D. pini* pheromones was shown to lead to reduced egg survival [20]. If this effect of pheromone exposure also applies under natural conditions, we expect a close link between the sawfly's oviposition preference and egg survival rate, even in forests.

Exposure of pine to *D. pini* pheromones is known to upregulate the expression of *PsRboh* (a pine respiratory burst oxidase homolog), indicating ROS signaling that may initiate further defense responses [50,51]. So far, we do not know whether pine changes its secondary metabolite profile or modifies its pattern of nutritious compounds in response to the exposure to *D. pini* pheromones. However, if so, *D. pini* larvae seem to be well adapted to such changes, since neither their weight nor their pupation success were affected when feeding upon pine trees that had been exposed to the pheromones (Figures 3 and 4).

4.4. No Amplifier Effect of Pheromone Exposure on Egg-Mediated Improved Anti-Herbivore Defense

While exposure of pine to *D. pini* pheromones did not affect larval performance, our study showed that larvae gain less weight when feeding upon previously egg-laden pine (Figure 3). These results, obtained with sawfly larvae feeding upon juvenile pine trees, corroborate previous findings which also showed that *D. pini* performs worse when feeding upon previously egg-laden branches of mature (at least 15-year-old) pine trees [27]. Hence, our findings suggest that pine can take *D. pini* egg deposition as a highly reliable indication of impending larval herbivory, rendering any amplification of the egg-mediated improvement of pine defense against larvae via a further response to the pheromones redundant.

5. Conclusions and Perspectives

Our study provided further insights into how the sawfly *D. pini* can adjust its behavior based on the responses of its host plant to cues preceding and indicating impending infestation. We showed here that *D. pini* females are reluctant to lay their eggs on pheromone-exposed pine, thereby escaping from the enhancer effect of pheromone exposure on pine defense against sawfly eggs. In contrast, larval performance was not affected by exposure of pine to sawfly pheromones. Future studies need to elucidate whether pine does not change its nutritive quality in response to the pheromones or whether larvae are well adapted to such potential changes. Furthermore, a comparison of the odor profiles of pheromone-exposed and unexposed pine needs to elucidate whether pine changes its volatile profile in response to the exposure to pheromones. Studies on the sawfly's olfactory perception of pine volatiles will further enhance our understanding of their behavior.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/insects15060458/s1, Table S1. Comparison of pupation of *Diprion pini* larvae that fed upon either egg-free or egg-laden *Pinus sylvestris* trees exposed to different pretreatments with volatiles. Pupation of larvae that fed on pine trees without egg deposition: untreated (U + L), hexane-treated trees (H + L), or pheromone-treated trees (P + L). Pupation of larvae that fed on trees with sawfly egg deposition (E): untreated (U + E + L), hexane-treated (H + E + L), or pheromone-treated (P + E + L) trees. Data: Mean (± SE) number of pupae per tree; each treatment

consisted of 7–10 trees and a maximum of 5 larvae per tree. Statistical differences were determined using the Mann–Whitney *U* test.

Author Contributions: Conceptualization, A.R.-S. and M.H.; methodology, A.R.-S. and M.H.; software, A.R.-S.; validation, A.R.-S.; formal analysis, A.R.-S.; investigation, A.R.-S. and N.B. (Figure 1); resources, A.R.-S. and M.H.; data analysis, A.R.-S.; writing—original draft preparation, A.R.-S.; writing—review and editing, A.R.-S. and M.H.; visualization, A.R.-S.; supervision, M.H.; project administration, M.H.; funding acquisition, M.H. All authors have read and agreed to the published version of the manuscript.

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Chapter 2: Supplement

Pine Response to Sawfly Pheromones: Effects on Sawfly's Oviposition and Larval Growth

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Table S1. Comparison of pupation *of Diprion pini* larvae that fed upon either egg-free or egg-laden *Pinus sylvestris* trees exposed to different pre-treatments with volatiles. Pupation of larvae that fed on pine trees without egg deposition: untreated (U+L), hexane-treated trees (H+L), or pheromone-treated trees (P+L). Pupation of larvae that fed on trees with sawfly egg deposition (E): untreated (U+E+L), hexane-treated (H+E+L), or pheromone-treated (P+E+L) trees. Data: Mean (± SE) number of pupae per tree; each treatment consisted of 7-10 trees and maximum 5 larvae per tree. Statistical differences were determined using the Mann Whitney *U* test.

	Treatments					
	U+L	**U+E+L**	**H+L**	**H+E+L**	**P+L**	**P+E+L**
Mean	4.00	3.27	3.80	3.54	3.70	3.00
SE	0.235	0.406	0.290	0.340	0.366	0.330
***p* value**	0.2111		0.6402		0.1015	

Chapter 3: Sawfly Sex Pheromones: Analysis of Their Impact on Pine Odor Attractive to Egg Parasitoids

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RESEARCH

Sawfly Sex Pheromones: Analysis of Their Impact on Pine Odor Attractive to Egg Parasitoids

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Abstract
Pinus sylvestris trees are known to efficiently defend themselves against eggs of the herbivorous sawfly *Diprion pini*. Their direct defense against eggs is primable by prior exposure to the sex pheromones of this species and their indirect defense involves attraction of egg parasitoids by egg-induced pine needle odor. But it is unknown whether exposure of pine to *D. pini* sex pheromones also affects pine indirect defense against sawfly eggs. In this study, we investigated the influence of exposure of *P. sylvestris* trees to the sex pheromones of *D. pini* on indirect defense mediated by egg parasitoids. Behavioral assays with *Closterocerus ruforum*, a key parasitoid of sawfly eggs, revealed no significant attraction to odor from egg-free pines pre-exposed to pheromones. Chemical analyses of odor from egg-free pines showed no pheromone-induced change in the emission rates of the known key terpenoids promoting parasitoid attraction. Further comparative analyses of odor from egg-laden pines pre-exposed to the sex pheromones and of odor from egg-laden pines unexposed to pheromones neither revealed significant differences in the emission rates of terpenoids relevant for parasitoid attraction. The results suggest that a pheromone-induced or pheromone-primed, egg-induced pine indirect defense seems to be redundant in addition to the known pheromone-primable pine direct defense against the eggs and the known egg-inducible indirect defense.

Keywords Pine · Sawfly · Egg parasitoids · Semiochemicals · Tritrophic interaction

Introduction

Plants emit volatile organic compounds (VOCs) that influence a myriad of interactions in trophic networks (Dicke 2016; Turlings and Erb 2018; Kessler et al. 2023; Kutty and Mishra 2023; Schuman 2023). Of these interactions, the attraction of parasitoids to plant VOCs emitted in response to insect herbivory or egg deposition has been intensively studied with respect to so-called plant indirect anti-herbivore defenses (Vet and Dicke 1992; Hilker and Fatouros 2015; Aljbory and Chen 2018; Ali et al. 2023; Gómez-Cabezas et al. 2023).

Certain plant volatile carboxylic acid esters, such as (*Z*)-3-hexenyl acetate, methyl jasmonate and methyl salicylate, have been recognized as inducers or priming agents of plant defenses against herbivorous insects (Engelberth et al. 2004; Frost et al. 2008; Kegge et al. 2013; Mageroy et al. 2020). Carboxylic acid esters are also prominently released as sex pheromones by a wide variety of herbivorous insect species (Francke and Schulz 2010; Naka and Fujii 2020). Notably, pheromones ranging from simple alkyl esters to more complex diesters have been identified across various species (Tabata et al. 2012; Tabata and Ichiki 2017; Meier et al. 2020). While a wide range of studies have investigated how plant VOCs influence the emission and perception of insect sex pheromones (Reddy and Guerrero 2004; Arx et al. 2012; Xu et al. 2017; Borrero-Echeverry et al. 2018; Hoffmann et al. 2020), little is known about how insect sex pheromones affect the emission of plant VOCs, which in turn may influence the behavior of herbivorous insects and their antagonists.

The sex pheromone components released by female *Diprion pini* sawflies are carboxylic acid esters, specifically (*2S*,*3R*,*7R*)-3,7-dimethyl-2-tridecanyl acetate and propionate (Bergström et al. 1995; Anderbrant et al. 2005). These chemical signals possess the remarkable ability to traverse great distances, effectively serving as beacons that

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can allure males (Anderbrant et al. 1995, 2005). Previous studies have demonstrated that the pheromones of *D. pini* are attractive to the eulophid egg parasitoid *Closterocerus ruforum* (Hilker et al. 2000), a species specialized on eggs of pine-infesting diprionid sawflies (Pschorn-Walcher and Eichhorn 1973; Eichhorn and Pschorn-Walcher 1976). The pheromones of the sawfly may serve as an early indication for the egg parasitoid that *D. pini* is present, potentially indicating the availability of host eggs. Moreover, *C. ruforum* females are attracted by the egg-induced pine odor. *Pinus sylvestris* twigs carrying three-day-old *D. pini* eggs on their needles emit enhanced quantities of (*E*)-β-farnesene, which, in combination with four other non-induced pine terpenoids is attractive to the parasitoid (Mumm et al. 2003; Beyaert et al. 2010).

Interestingly, *D. pini* sex pheromones have been shown to prime pine trees' direct defenses against sawfly eggs. Sawfly eggs laid on pheromone-exposed pine needles suffer lower survival rates compared to eggs on unexposed trees (Bittner et al. 2019). This priming activity of the *D. pini* pheromones on pine direct defenses against sawfly eggs raised the question of whether the pheromones also impact pine indirect defenses involving egg parasitoid attraction.

Here, we studied (i) whether exposure of egg-free pine trees to *D. pini* pheromones can induce *P. sylvestris* VOCs attractive to these parasitoids and/or (ii) whether the pheromones can prime the egg-induced emission of (*E*)-β-farnesene, which attracts the egg parasitoid *C. ruforum* to egg-laden pine when combined with four other non-induced pine terpenoids.

We addressed the question on the inductive capability of *D. pini* pheromones on parasitoid-attracting pine VOCs by comparing, first, the parasitoids' behavioral response to odor from (egg-free) pheromone-exposed and unexposed pine trees. Furthermore, we chemically analyzed whether pheromone-exposed pine differs from unexposed pine by the emission rates of (*E*)-β-farnesene and the other pine VOCs that, in combination with the egg-induced quantities of (*E*)-β-farnesene, provide an odor attractive to the parasitoids (Beyaert et al. 2010). In addition, we investigated whether the exposure of pine to *D. pini* sex pheromones further enhances (primes) the egg-induced emission of (*E*)-β-farnesene and/or affects the emission of the other four pine VOCs relevant for attraction of *C. ruforum.*

By exploring the attraction of egg parasitoids to pheromone-exposed pine and the potential effects of the pheromones on pine VOCs, this study aims to contribute to our understanding of the complex network of chemical communication in the tripartite interactions among plants, herbivorous insects and parasitoids.

Methods and Materials

Plants

For the experiments, three-year-old *P. sylvestris* trees (40–60 cm height, 2–4 cm stem Ø) were purchased from a local tree nursery (Baumschule Stackelitz GmbH and Co. KG, 06868 Coswig / OT Stackelitz, Germany). Each tree grew individually in pots filled with Classic T potting soil (Einheitserde®, a mixture of peat and clay; $N = 340$ mg·L^{-1}, $P_2O_5 = 260$ mg·L^{-1}, $K_2O = 330$ mg·L^{-1}). Prior to the experiments, the trees were kept in a greenhouse under long-day conditions (20 °C, 18:6 h, L:D cycle). One week before starting the experiments, the trees were transferred to a climate chamber, where they could acclimate to the abiotic experimental conditions (20 °C, 18:6 h, L:D cycle, 70% RH, 100 µmol photons m^{-2}·s^{-1}). Each potted tree was kept in a transparent Plexiglas® cylinder (14.1 L, 80 cm height, 15 cm Ø). The cylinder was equipped with an inlet for charcoal-filtered air at the bottom (250 mL·min^{-1}) and an air outlet on top (250 mL·min^{-1}). Thus, the trees inside the cylinders were exposed to only charcoal-filtered air. The pots were wrapped with polyethylene terephthalate (PET) bags covering the pots and soil, thus preventing VOCs released from the soil or the roots from affecting aboveground pine responses.

Insects

Diprion pini were reared under laboratory conditions in a climate chamber (20 °C, 18:6 h, L:D cycle, 70% RH, 100 µmol photons m^{-2}·s^{-1}) following the protocol from Eichhorn (1976). Females laid eggs onto the needles of *P. sylvestris* branches collected from a forest southwest of Berlin. The eggs are laid in a row of about 10–15 eggs onto a pine needle. Each pine branch was provided with tap water and kept inside a transparent Plexiglas® cylinder (14.9 L, 50 cm height, 19.5 cm Ø), which was closed on top with a gauze lid. Larvae hatched from eggs about 12 to 14 d after egg deposition and started feeding on pine needles. Pupation began approximately three weeks after larval hatching. Cocoons were collected and stored at 4 °C. To initiate the emergence of adults, the cocoons were transferred back to the rearing climate chamber five days before use for the experiments. Following emergence, the adults were transferred to a separate climate chamber (10 °C, 18:6 h, L:D cycle, 70% RH, 100 µmol photons m^{-2}·s^{-1}) until required for the experiments.

The eulophid egg parasitoid *C. ruforum* was obtained from parasitized eggs of a close relative of *D. pini*, i.e. the pine sawfly *Neodiprion sertifer.* The parasitized eggs were collected in a Finnish forest, sent to our Berlin laboratory,

where they were stored in Petri dishes at 5 °C for 14 days at maximum. To initiate parasitoid emergence, we transferred the Petri dishes containing needles with parasitized eggs to 20 °C (18:6 h, L:D cycle, 70% RH). Emergence of adult parasitic wasps from the sawfly eggs typically started 8 to 10 days later. Upon emergence from host eggs, adult parasitoids were individually placed in a small Petri dish (3 cm Ø) and stored at 10 °C (18:6 h, L:D cycle, 70% RH). For feeding, GIZEH® filter papers (3 × 15 mm) soaked in a 15% aqueous honey solution were added to each dish. For bioassay preparation, females were allowed to mate at 20 °C. Therefore, a male was placed into each dish with a female for 24 h. Thereafter, female parasitoids were separated from the males and kept for an additional period of 24 h ('lag phase') under the conditions described for storage (see above). After the lag phase, the bioassays were conducted.

Treatment of Pine

Pine trees were exposed to synthetic male-attracting sex pheromones of *D. pini* females, specifically (*2S*,*3R*,*7R*)-3,7-dimethyl-2-tridecanyl acetate and propionate, supplied by the laboratory of Olle Anderbrant, Lund University, Sweden. We used the same pheromone concentrations as those known to prime pine direct defense against *D. pini* eggs; these concentrations are comparable to those which pine trees are exposed to during *D. pini* mass outbreaks (Bittner et al. 2019). Thus, the pheromone esters were each dissolved in hexane at a concentration of 50 ng·µL^{-1}. For treatment of the trees, 100 µL of the pheromone solution (50 µL (*2S*,*3R*,*7R*)-3,7-dimethyl-2-tridecanyl acetate + 50 µL (*2S*,*3R*,*7R*)-3,7-dimethyl-2-tridecanyl propionate) was applied to a cotton wool pad (5.6 cm Ø, 0.4 cm thickness) as the dispenser. To allow for solvent evaporation, the cotton pads were left under a fume hood for 30 min before being exposed to the trees. After solvent evaporation, the cotton pad with pheromones was placed into a Plexiglas® cylinder (14.1 L, 80 cm height, 15 cm Ø) with a pine tree for 24 h.

Because the pheromones were dissolved in hexane, a solvent control with 100 µL hexane only was also included. A pad was treated with only 100 µL hexane, placed under a fume hood for 30 min, and thereafter placed into a cylinder with a pine tree for 24 h.

A second control (blank) was provided by adding an untreated cotton pad without any solvent to a cylinder with a pine tree.

First, egg-free pine trees were subjected to the above-described treatments to investigate whether exposure of pine to the pheromones (or hexane) can induce a change in pine odor. We used $n = 5$–6 replicates per treatment.

In addition, to study whether exposure of pine to the pheromones (or hexane) affects the release of the known egg-induced pine volatiles, the above-described treatments were followed by *D. pini* egg depositions for 24–72 h, until each tree had received three to four egg rows on its needles. The successful egg laying was monitored at timely intervals. The insects were removed from the trees after the targeted number of egg masses had been laid. For this combined treatment by exposure to volatiles and subsequent oviposition, we used $n = 5$–8 replicates per treatment.

Olfactometer Bioassay with Egg Parasitoids

Olfactometer bioassays with parasitoids were conducted using a four-field olfactometer. The design of the olfactometer was similar to the setup described by Hilker et al. (2002). In short, odor from four sources flowed into four olfactometer fields at a rate of 155 ml·min^{-1}. To determine whether the egg parasitoid is attracted by odor released by pheromone-exposed pine, one field was ventilated with odor from a pheromone-treated pine, and the opposite field was ventilated either with odor from a hexane-treated pine or an untreated control pine. The remaining two fields were considered buffer fields, ventilated with charcoal-filtered air only. The parasitoids were exposed to odor from pheromone-exposed (or hexane-exposed) pine right after the end of the 24 h-exposure period, and also one and two days later.

After a parasitoid female was placed into the center of the olfactometer, its behavior was recorded for a period of 10 min using the software Observer 3.0 (Noldus, Wageningen, The Netherlands). We recorded for how long the parasitoids were actively moving around (walking) in the olfactometer field supplied with odor from pheromone-exposed pine compared to the opposite field and also in the buffer fields. The majority of the tested parasitoid females was actively moving in these olfactometer fields. We calculated the relative walking activity as percentage active walking time from total observation time (10 min; 100%) (for detailed information on the activity of the parasitoids in the fields supplied with odor from differently treated pine and in the buffer fields, see Supplemental Table S1). When offering the parasitoids the odor from a pheromone-treated tree and from an untreated (blank) pine in the opposing field, we tested in total $n = 9$ parasitoids/time point and $n = 3$ trees/treatment. When offering the odor from a pheromone-treated tree and a hexane-treated tree in the opposing field, we tested in total $n = 18$ parasitoids/time point and $n = 6$ trees/treatment.

Collection of Pine Volatiles

Pine VOCs were collected from pheromone-treated, hexane-treated and untreated (only cotton pad) *P. sylvestris* trees. One set of these trees was without sawfly egg

depositions, the other set received egg depositions after the treatment with pheromones (or hexane). The collection of pine volatiles was always conducted between 10 am and 11 am, during the first third of the daylight phase in the climate chamber (20 °C, 18:6 h, L:D cycle, 70% RH, 100 µmol photons $m^{-2} \cdot s^{-1}$).

To investigate possible changes in emission rates of volatiles in the course of time post treatments, we collected pine VOCs at different time points. The headspace of each egg-free tree was sampled at four time points: directly (0 h) post exposure, and 24 h, 48 h, and finally 72 h post pheromone or hexane exposure. The headspace of egg-laden trees was also collected at four time points, i.e. 24 h, 48 h, 72 h, and 96 h after the end of the oviposition period.

The VOCs emitted from the differently treated pine trees were collected on Tenax® TA filters. Prior to VOC collection, the filters were conditioned at 280 °C. Filters were integrated into the cylinder's outlet airflow, and collection was conducted at a rate of 100 $mL \cdot min^{-1}$ for 1 h with an inflow of charcoal-filtered air maintained at 200 $mL \cdot min^{-1}$ from the bottom. After the trapping of volatiles, they were always analyzed on the same day.

Chemical Analysis of Pine Terpenes

The headspace volatiles from pine trees were analyzed using gas chromatography-mass spectrometry (7890 A and 5975 C VL MSD, Agilent, Waldbronn, Germany). Trapped pine VOCs were desorbed from the filter in a thermal desorption unit (TDU) (GERSTEL, Mülheim, Germany). For later quantification of the pine VOCs, 2.5 µL methyl octanoate (50 $ng \cdot \mu L^{-1}$) were injected as internal standard (IS) into each Tenax® TA filter with pine VOCs; this was done just prior to introducing the filter into the TDU. The TDU started at 30 °C and then heated up at a rate of 100 °C min^{-1} until it reached 290 °C, where it was held for 3 min. Subsequently, the VOCs were concentrated in a Programmable Temperature Vaporization (PTV) type inlet (CIS 4, Gerstel) at -50 °C. The temperature of the cryotrap then increased from − 50 °C to 290 °C at a rate of 12 °C sec^{-1} to transfer the VOCs to the column. The VOCs were transferred in splitless mode to an HP5-MS column UI capillary column (30 m x 250 µm x 0.25 µm) with helium as a carrier gas at a flow rate of 1 $mL \cdot min^{-1}$. The front PTV inlet for helium was set to solvent vent mode, with a purge flow to split vent set at 5 $mL \cdot min^{-1}$ starting at 0.01 min. The temperature programming of the oven included two stages: initially set at 40 °C for 5 min, the temperature increased at a rate of 5 $°C \cdot min^{-1}$ to reach 260 °C without any holding period, followed by a sharper increase at 60 $°C \cdot min^{-1}$ up to 300 °C, which was then sustained for 10 min. Mass spectra of the VOCs were obtained by electron impact ionization at 70 eV (scan mode range 33 to 350 *m/z*).

The analysis of the trapped pine volatiles focused on those five terpenoids that are known to be crucial for the positive (electrophysiological and behavioral) response of the egg parasitoid *C. ruforum* to egg-induced pine odor (Beyaert et al. 2010). These compounds — β-phellandrene, (*E/Z*)-β-ocimene, β-caryophyllene, α-humulene, and (*E*)-β-farnesene — are collectively effective in attracting *C. ruforum* when mixed in the same ratios as emitted from egg-laden pine. While the emission of (*E*)-β-farnesene is induced by *D. pini* egg deposition (Mumm et al. 2003; Mäntyla et al. 2018), the emission of the four other compounds was so far not found to be affected by *D. pini* egg deposition, but necessary to be combined with the egg-induced emission rate of (*E*)-β-farnesene for attraction of *C. ruforum* (Beyaert et al. 2010).

For identification of these terpenoids, authentic standards of each compound were acquired from the following sources: (*E/Z*)-β-ocimene and (*E*)-β-farnesene from Sigma-Aldrich (Taufkirchen, Germany), β-phellandrene from Biomol (Hamburg, Germany), β-caryophyllene and α-humulene from Fluka (Fluka / Fisher Scientifics, Schwerte, Germany) and methyl octanoate from TCI (Eschborn, Germany). Pine VOCs were identified by comparison of their mass spectra and retentions indices with those of authentic standards.

For quantification, standard samples with known concentrations (1, 2.5, 5, 10, 25, 50 and 100 $ng \cdot \mu L^{-1}$) were analyzed under identical GC-MS conditions as the pine tree headspace samples by injecting an aliquot of 2.5 µL into a Tenax® TA filter. We measured the peak areas of the compounds in total ion current chromatograms (TICs). To cope with potential measurement variability over time (Noonan et al. 2018), peak areas of the standard samples were normalized against the internal standard (methyl octanoate), which had been injected into the Tenax® TA filter as well (injection volume: 2.5 µL; concentration: 50 $ng \cdot \mu L^{-1}$). Dose-response curves (concentration standard sample vs. normalized peak area) were generated using linear regression models. Peak areas of pine VOCs were also normalized against the IS peak area. Some pine VOCs were detected with relative peak areas smaller than the ones measured at the lowest concentration of the standard runs. For example, the mean of the smallest measured relative areas of α-humulene as standard sample (1 $ng \cdot \mu L^{-1}$) was 0.11 (11% of the measured area of the IS peak). Therefore, if a pine VOC sample had a relative area less than this, it was not approximated with the curve provided by the linear regression analysis, but rather with a curve that goes from the lowest measured point through the origin.

On the HP5-MS column, β-phellandrene was not separated from limonene and was therefore calculated by the following formula as described by Mumm et al. (2003):

$$X = \frac{A_{68} \cdot \frac{C_{93}}{B_{68}}}{A_{68} \cdot \frac{C_{93}}{B_{93}} + (A_{93} - A_{68} \cdot \frac{C_{93}}{B_{68}} \cdot \frac{B_{93}}{B_{68}})} \quad Y = 1 - X$$

The variables stand for the following. Y: portion of β-phellandrene in total ion current chromatogram (TIC)-peak of β-phellandrene + limonene, X: portion of limonene in the TIC-peak of β-phellandrene + limonene, B_{68}: portion of mass 68 of the total ion yield of a limonene pure sample (%), A_{68}: peak area of ion trace 68; A_{93}: peak area of ion trace 93, B_{93}: portion of mass 93 of the total ion yield of a limonene pure sample (%), and C_{93}: portion of mass 93 of the total ion yield of a β-phellandrene pure sample (%).

Statistical Analysis

Graphing and data analysis were performed using Prism version 10.2.1 (GraphPad Software). Initially, data normality was assessed using the Shapiro-Wilk method, followed by appropriate statistical tests. P-values greater than 0.05 were considered not significant (ns), while p-values below 0.05 were deemed significant.

For the behavioral response data of the egg parasitoid, the Wilcoxon matched-pairs signed rank test was used to compare parasitoid activity levels in different olfactometer fields.

For the emission rates of pine key terpenoids, differences among the treatment groups were determined using the Kruskal-Wallis test by comparing emission rates from (i) untreated, hexane-exposed, pheromone-exposed pine without egg deposition among each other and (ii) from untreated, hexane-exposed, pheromone-exposed pine with egg deposition among each other. Additionally, a Mann-Whitney *U* test was performed to further evaluate the impact of *D. pini* egg deposition on emission rates of the analyzed terpenoids from the young trees used here (comparison of odor from egg-free pine and odor from egg-laden pine, both without exposure to pheromones).

Results

Olfactometer Bioassay with Egg Parasitoids

The olfactometer bioassays showed that the egg parasitoid *C. ruforum* is not attracted by odor from pheromone-exposed pine when compared to odor from untreated pine (Fig. 1a) or to odor from hexane-exposed pine (Fig. 1b). The time during which the parasitoid females walked around in the olfactometer field supplied with odor from pheromone-exposed pine did not significantly differ from the time in the opposing field supplied with odor from untreated or hexane-exposed pine (For details on parasitoid activity in fields with odor from treated pine and buffer fields, see Supplemental Table S1). This lack of preference for pheromone-exposed pine was consistent across the different time points post exposure.

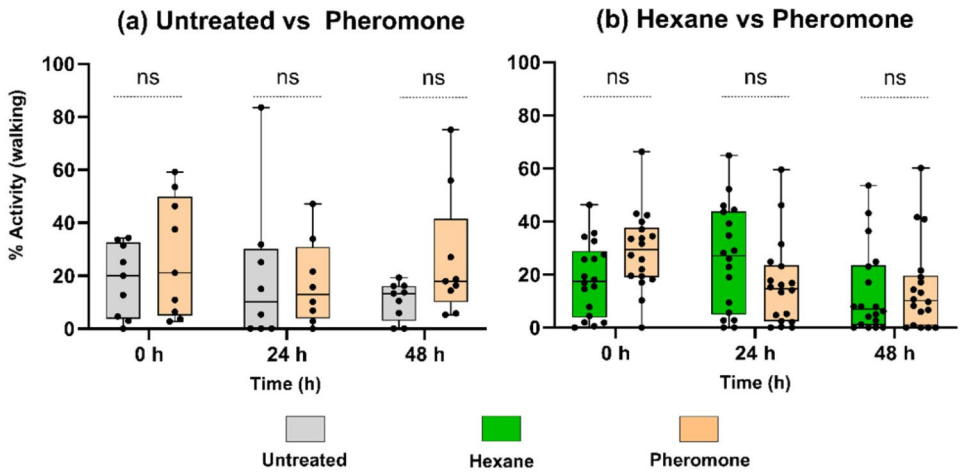

Fig. 1 Behavioral response of the egg parasitoid *Closterocerus ruforum* to odor from *Pinus sylvestris* trees 0, 24, and 48 h post exposure to either *Diprion pini* pheromones or hexane or were left untreated. The activity level of the parasitoids was measured by recording the time they spent walking in the olfactometer field that was supplied with the odor from pheromone-exposed trees and in the opposite field with odor from untreated trees (**a**) or trees exposed to hexane (**b**). The total observation lasted for 10 min. Data expressed as percentage active walking time from total observation time (100%). The whiskers represent the minimum and maximum values, and the middle line inside boxes denotes the median, (**a**) $n = 9$ parasitoids/time point; $n = 3$ trees/treatment, (**b**) $n = 18$ parasitoids/time point; $n = 6$ trees/treatment. Statistical analysis: Wilcoxon matched-pairs signed rank test; ns not significant ($p > 0.05$)

Chemical Analysis of Pine Terpenes

The headspace of differently treated *P. sylvestris* trees was analyzed with respect to the emission rates of five key volatile components, which are known to attract the egg parasitoid *C. ruforum* (Beyaert et al. 2010). Among these five components, emission rates of both stereoisomers (*E* and *Z*) of β-ocimene were included. Headspace samples were collected directly post treatment (0 h) and at intervals of 24, 48, and 72 h post treatment from trees exposed to *D. pini* pheromones, hexane, or from trees that were left untreated. The GC-MS analysis revealed no significant differences in the emission rates of these substances across the treatments at any time sampling point (Fig. 2, Supplemental Table S2).

To test whether the exposure of pine to *D. pini* pheromones affects the emission rate of egg-induced pine, we analyzed in a follow-up study the emission rates from *P. sylvestris* first exposed to pheromones (or hexane) and

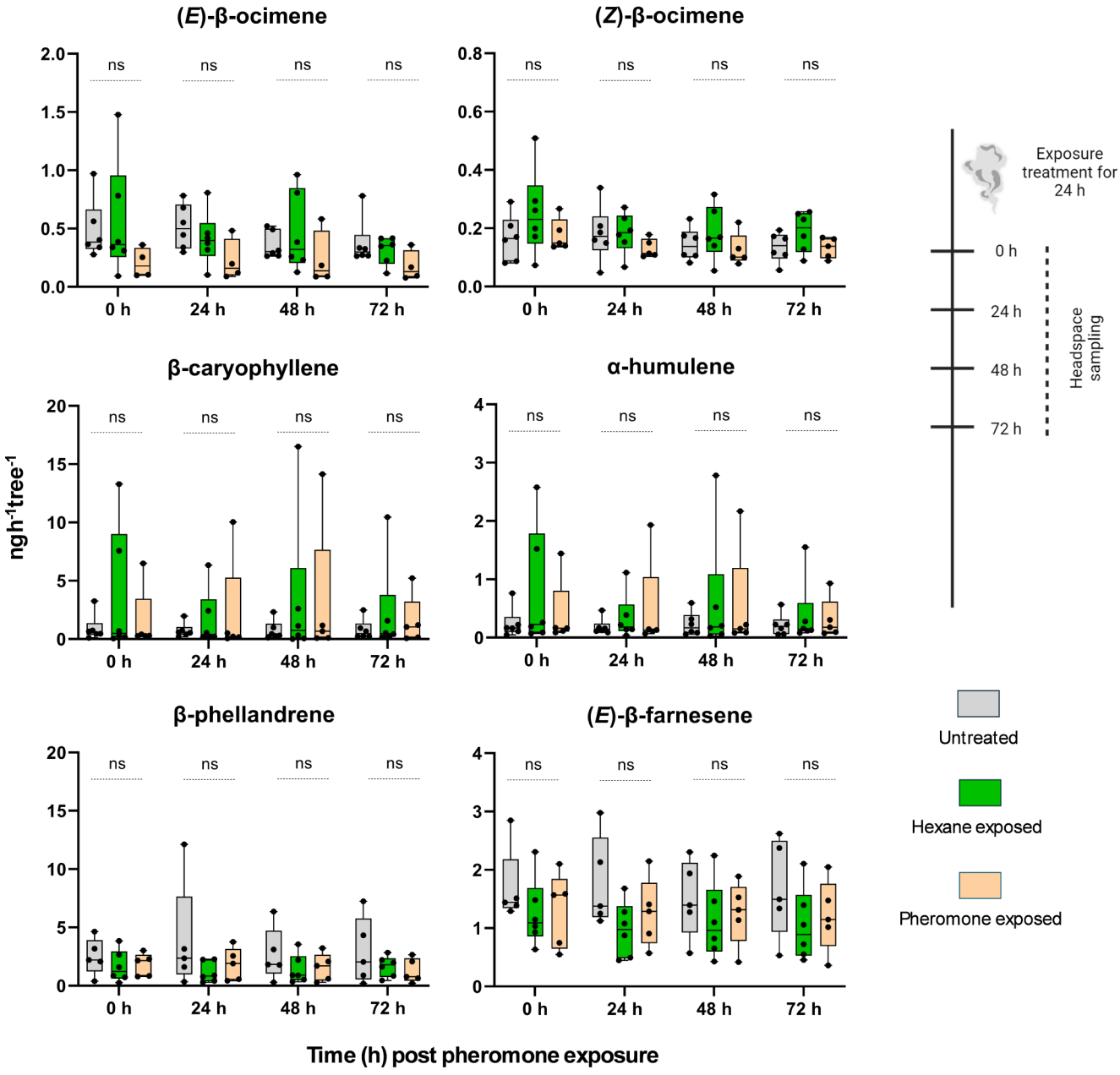

Fig. 2 GC-MS analyses of the odor from differently treated, egg-free *Pinus sylvestris* trees. Emission rates of pine key terpenoids that are known to attract the egg parasitoid *Closterocerus ruforum* were recorded. Trees were left untreated or were exposed to hexane or *Diprion pini* pheromones. Headspace samples were collected repeatedly from each tree at four different time intervals after the end of pine treatments: 0, 24, 48, 72 h. The whiskers represent the minimum and maximum values, and the middle line inside the boxes denotes the median ($n = 5$–6 trees per treatment). Statistical analysis: Kruskal-Wallis test; ns not significant ($p > 0.05$)

subsequently subjected to *D. pini* egg deposition. Again, we focused our analysis on those five key terpenoids that are known to be relevant for attraction of the egg parasitoid *C. ruforum*. The emission rates of (*E*)-β-farnesene and (*E*)-β-ocimene released from previously pheromone-exposed, egg-laden pine were by trend higher than those released from control trees (Fig. 3). However, our analyses revealed no significant differences in the emission rates of these compounds when comparing the pheromone-treated, egg-laden pine with the respective egg-laden control pine trees (treated with hexane or left untreated) by a Kruskal-Wallis test (Fig. 3, Supplemental Table S2).

To test whether the young pine trees used in our study respond to *D. pini* egg deposition as known for branches of mature pine trees (Mumm et al. 2003; Mäntyla et al. 2018), we compared the emission rates of the analyzed terpenoids

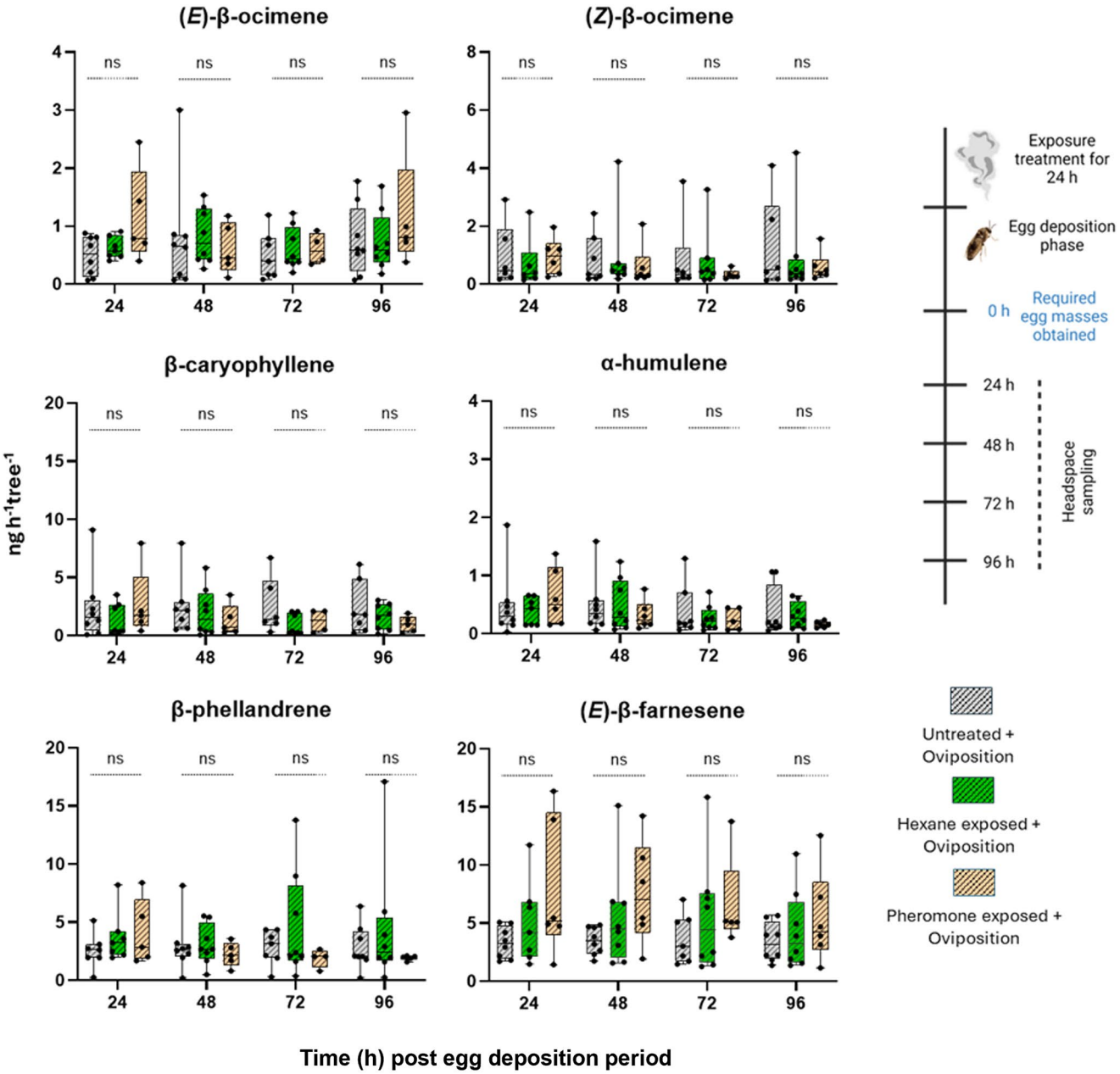

Fig. 3 GC-MS analysis of differently treated *Pinus sylvestris* trees laden with *Diprion pini* eggs. Emission rates of pine key terpenoids that are known to attract the egg parasitoid *Closterocerus ruforum* were recorded. Trees were left untreated or exposed to hexane or *D. pini* pheromones, followed by egg deposition by *D. pini* females. Headspace samples were collected repeatedly from each tree at four different time intervals after the end of the egg deposition period: 24, 48, 72 h, and 96 h. The whiskers represent the minimum and maximum values, and the middle line inside the boxes denotes the median ($n = 5$–8 trees per treatment). Statistical analysis: Kruskal-Wallis test; ns not significant ($p > 0.05$)

from untreated pine and from pine with egg deposition. In accordance with the response of mature trees to *D. pini* egg deposition, the egg-laden, young pine trees emitted significantly higher quantities of (*E*)-β-farnesene than egg-free pine (Supplemental Table S3). Interestingly, the egg-laden pine trees studied here emitted also higher quantities of (*Z*)-β-ocimene than egg-free ones, whereas mature trees did not show a significantly egg-induced emission of this terpenoid compound (Supplemental Table S3).

Discussion

Previous research showed that exposure of *P. sylvestris* to *D. pini* sex pheromones primes pine direct defenses against sawfly eggs and leads to decreased egg survival rates (Bittner et al. 2019). Here, we investigated whether exposure of pine to the sawfly's pheromones also impacts pine indirect defenses against the eggs. Studies of the behavioral responses of the egg parasitoid *C. ruforum* revealed that exposure of pine to the pheromones did not induce a pine odor that is attractive to this antagonist of the sawfly eggs. Our comparative chemical analyses of the headspace of pheromone-exposed and control pines revealed no significant differences in the emission rates of those terpenoids that are known to be relevant for the attraction of the parasitoid (Beyaert et al. 2010). We furthermore, investigated whether exposure of pine to the pheromones can prime the egg-inducible emission rate of (*E*)-β-farnesene, which is known to attract the parasitoid when combined with four further pine terpenoids that are not egg-induced (Beyaert et al. 2010). However, comparative chemical analyses of egg-laden pines exposed to pheromones and those unexposed showed no significant difference in the emission rate of (*E*)-β-farnesene.

The egg parasitoid *C. ruforum* is known to be attracted to the odor from pine induced by host egg deposition, but not to the odor from untreated, egg-free host plants (Hilker et al. 2002). Our finding that the egg parasitoids were not attracted to pheromone-exposed pines (Fig. 1) matches the lack of any notable pheromone-induced change in the emission rate of those pine volatiles relevant for attraction of *C. ruforum* (Fig. 2). Furthermore, the lack of attraction of *C. ruforum* by odor emitted from pheromone-exposed pine supports a previous study by Bittner et al. (2019), which showed that young *P. sylvestris* trees that had been exposed to *D. pini* pheromones as done in our study do not release any pheromonal traces after a 24-h exposure period. In accordance with this previous finding, the egg parasitoid *C. ruforum*, which is known to be attracted by *D. pini* pheromones (Hilker et al. 2000), did not prefer the odor from pine exposed to *D. pini* pheromone to odor from unexposed pine.

Since we tested parasitoids collected in forests from eggs of *Neodiprion sertifer*, but not from *D. pini* eggs, it cannot be excluded that the lack of attraction by odor from pine exposed to *D. pini* pheromone was due to local adaptation of the tested individuals to their natal host species, its pheromone and the impact of the *N. sertifer* pheromone on the host plant. The pheromone of *N. sertifer* has been identified as (2*S*, 3*S*, 7*S*)-3,7-dimethyl-2-pentadecanol esterified with acetic acid (Jewett et al. 1976; Wassgren et al. 1992), thus differing from the *D. pini* pheromone by its stereochemistry and the chain length of the alcohol component of the ester. Especially in parthenogenetically reproducing parasitoids, local adaptation to the environment may facilitate maintenance of within-species genetic variance and support successful development in host species of different quality (Godfray 1994; Harvey et al. 2012; Hopper et al. 2019; Harrison et al., 2022). In our previous studies, which showed attraction of *C. ruforum* to *D. pini* sex pheromones (Hilker et al. 2000) and to pine odor induced by *D. pini* eggs (Hilker et al. 2002; Schröder et al. 2008), we also used individuals collected from *N. sertifer* eggs at the same locations in Finland as the parasitoids used in our current study. These previous results showed that those previously collected *C. ruforum* individuals, which developed in *N. sertifer* eggs, can respond to infochemicals associated with *D. pini.*

Trees exposed to pheromones of herbivorous insects face the challenge of responding to cues that do not indicate the precise location of the attacker since a pheromone plume may widely disperse within the tree canopy. The attraction of *C. ruforum* to *D. pini* sex pheromones (Hilker et al. 2000) might help the parasitoid in habitat location when orientating towards the odor source, while the attraction to egg-induced pine odor (Hilker et al. 2002) can help the parasitoid locate egg-laden needles. If exposure of egg-free pine to *D. pini* sex pheromones were to induce an odor attractive to the parasitoid, this might interfere with these known attractive odors, and thus might be no beneficial pine response to the sex pheromones of its attacker.

However, if exposure of pine to *D. pini* sex pheromones would prime the emission rate of egg-inducible (*E*)-β-farnesene, such a priming effect could enhance the attraction of *C. ruforum* and guide more parasitoids to egg-laden pine needles. Interestingly, the data show that shortly after the end of the oviposition period, the emission rates of especially (*E*)-β-ocimene and (*E*)-β-farnesene tended to be higher in previously pheromone-exposed, egg-laden pine than in egg-laden pine without prior pheromone exposure. However, our chemical analyses found no evidence for a significant priming effect of *D. pini* pheromones on the egg-inducible emission rate of (*E*)-β-farnesene. Thus far, there is no indication that an increase in the emission rate of (*E*)-β-ocimene is relevant for attraction of *C. ruforum.* This

egg parasitoid species is highly sensitive to (*E*)-β-farnesene and is attracted by it only when combined with four other pine terpenes – among them (*E*)-β-ocimene – which are not inducible by *D. pini* egg deposition per se (Mumm et al. 2003; Beyaert et al. 2010). Future studies should elucidate the ecological relevance of the enhanced emission rate of (*E*)-β-ocimene in pheromone-exposed, egg-laden pine compared to pheromone-exposed, egg-free pine.

Our study corroborates previous findings (Mumm et al. 2003; Mäntyla et al. 2018), which showed that *D. pini* egg deposition induces the emission of (*E*)-β-farnesene, a key terpene for the attraction of the egg parasitoid *C. ruforum*. In previous studies, attraction of this parasitoid by egg-induced pine odor and the enhanced emission rate of (*E*)-β-farnesene were found 72 h after *D. pini* egg deposition (Hilker et al. 2002; Mumm et al. 2003; Schröder et al. 2008). In contrast to these previous studies, we found (i) that also (*Z*)-β-ocimene was induced by the sawfly egg deposition and (ii) that (*E*)-β-farnesene is not only induced 72 h post egg deposition, but already earlier after 24 h (Supplemental Table S3). These differences may be due to different developmental stages of pine used for our study and the previous ones. Unlike previous studies that analyzed the response of pine twigs from mature trees to sawfly egg deposition, we sampled volatiles from egg-laden, three-year-old pine saplings. Our findings suggest that pine saplings respond more swiftly to egg deposition by *D. pini* and accelerate the release of VOCs, which are crucial for attracting antagonists of the eggs. This finding is supported by previous molecular studies of *P. sylvestris* laden with *D. pini* eggs. A recent transcriptomic analysis of pine saplings laden with eggs of *D. pini* showed upregulation of genes involved in terpene biosynthesis as soon as one hour post egg deposition (Hundacker et al. 2024). In contrast, a previous analysis of twigs taken from mature trees revealed that transcription rates of the sesquiterpene synthases *PsTPS* 1 and 2 (encoding (*E*)-β-caryophyllene and α-humulene for *PsTPS* 1, and 1(10),5-germacradiene-4-ol for *PsTPS* 2) were only enhanced 72 h after *D. pini* egg deposition (Köpke et al. 2008).

Taken together, no significant effects of the exposure of pine to *D. pini* sex pheromones on pine indirect defenses against the sawfly eggs were detected in our study. In contrast, *D. pini* sex pheromones were previously shown to affect (prime) pine direct defenses against the eggs, thus providing evidence for the responsiveness of pine to these insect volatiles (Bittner et al. 2019). Plant indirect and direct defenses have often been discussed and studied regarding trade-offs due to resource allocation costs (Cipollini et al. 2003; Agrawal and Fishbein 2006; Agrawal 2011). An indirect, pheromone-elicited defense of pine against the infestation with *D. pini* eggs might be too costly, as it would be in addition to the known egg-inducible, indirect defense response Hilker et al. 2002; Bittner et al. 2019) and the pheromone-primable, direct defense against eggs (Bittner et al. 2019). We suggest that the attraction of egg parasitoids to *D. pini* sex pheromones and egg-induced pine odor renders a pheromone-induced or pheromone-primed, egg-induced 'cry for help' (Dicke et al. 1990) redundant. The benefits of a defensive plant response to insect infestation are expected to, at least, balance or, at best outweigh the costs for the defense (Pearse et al. 2020). If the attraction of *C. ruforum* by *D. pini* sex pheromones and egg-induced pine odor provides sufficient protection of pine trees, any further investment of pine into additional signaling might represent an unnecessary energy expenditure.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10886-024-01547-1.

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Author Contributions ARS and MH conceived and designed the study. ARS treated the plants with pheromone and insects. MV collected the egg parasitoids. LS and ARS performed the olfactometer bioassays. ARS conducted the headspace sampling from trees. LS, ARS, and AR evaluated the GC-MS data. ARS conducted the statistical analyses. ARS wrote the first draft of the manuscript, ARS and MH wrote revised versions, and all authors contributed to the final version of the manuscript.

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Data Availability Data will be made available upon request.

Declarations

Ethical Approval Not applicable.

Competing Interests The authors declare no competing interests.

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Chapter 3: Supplement

Sawfly Sex Pheromones: Analysis of Their Impact on Pine Odor Attractive to Egg Parasitoids

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Supplemental Table S1: Detailed information about the walking activity of the egg parasitoid *Closterocerus ruforum* in four-field olfactometer assays (compare Fig. 1, main manuscript). The walking activity was recorded when testing the parasitoids' response to odor from *Pinus sylvestris* trees 0, 24, and 48 h post exposure to either *Diprion pini* pheromones or hexane or were left untreated. Tested: Odor from pheromone-exposed tree versus untreated tree (left table); odor from pheromone-exposed tree versus hexane-treated tree (right table); for each test: two buffer fields. Data show (i) relative walking time (%) of total observation period (10 min = 100%) for each tested parasitoid individual in each olfactometer field, (ii) the "total (sum)" of the relative walking times for each parasitoid individual in all four olfactometer fields, and (iii) the overall mean walking activity ± SE of all parasitoids when studying their response to "pheromone vs untreated" and when studying their response to "pheromone vs hexane" (calculated from "total (sum)" data).

pheromone vs untreated						
time	pheromone	untreated	buffer 1	buffer 2	total (sum)	mean walking activity ± SE
0 h	10.91	33.58	23.3	32.2	99.99	75.9 ± 6.24
	21.1	34.31	19.67	24.91	99.99	
	3.62	20	0.52	74.16	98.3	
	53.55	0	0.43	45.4	99.38	
	2.73	25.16	18.54	52.88	99.31	
	6.4	31.44	5.1	54.99	97.93	
	59.23	12.72	11.47	16.3	99.72	
	46.27	4.6	48.85	0	99.72	
	37.52	3.05	23.42	17.42	81.41	
24 h	10.21	31.8	15.75	41.94	99.7	
	15.73	25.09	5.5	24.01	70.33	
					0	
	2.9	0	0	20.91	23.81	
	47.14	0	33.33	0	80.47	
	6.85	83.58	0	5.81	96.24	
	33.99	0	6.32	15.02	55.33	
	0	5.39	7.61	0	13	
	21.59	15.04	37.91	0.5	75.04	
48 h	16.36	13.25	28.25	35.72	93.58	
	14.37	13.7	13.14	44.93	86.14	
	18.72	16.19	38.69	26.11	99.71	
	27.06	5.92	0	5.25	38.23	
	5.75	19.3	0	27.36	52.41	
	17.97	10.51	49.37	18.88	96.73	
	75.15	0	9.36	4.49	89	
	55.92	16.14	8.29	19.59	99.94	
	5.32	0	0	0.6	5.92	

pheromone vs hexane						
time	pheromone	hexane	buffer 1	buffer 2	total (sum)	mean walking activity ± SE
0 h	18.19	35.63	28.73	17.44	99.99	74.4 ± 4.26
	33.46	34.22	0	27.16	94.84	
	17.06	32.66	15.16	35.03	99.91	
	19.52	0.52	6	55.25	81.29	
	34.45	46.19	4.54	5.96	91.14	
	10.33	14.58	9.75	19.49	54.15	
	42.98	0	29.08	26.6	98.66	
	33.65	1.86	0	52.54	88.05	
	31.66	15.58	1.03	51.28	99.55	
	27.33	19.28	1.35	33.27	81.23	
	0	25.7	10.42	14.61	50.73	
	0	1.23	0	0	1.23	
	39.86	25.84	10.87	22.82	99.39	
	42.38	16.85	13.51	26.23	98.97	
	25.67	18.14	27.35	16.47	87.63	
	66.33	1.95	4.86	26.66	99.8	
	19.37	27.61	8.77	43.94	99.69	
	21.94	7.82	30.85	15.88	76.49	
24 h	13.38	64.9	3	7.78	89.06	
	0	0	0	5.6	5.6	
	31.44	18.97	38.76	10.82	99.99	
	4.71	43.65	11.79	25.87	86.02	
	0.28	45.98	23.46	16.55	86.27	
	2.13	34.71	34.13	22.55	93.52	
	17.71	39.19	20.03	20.73	97.66	
	2.49	22.87	0	24.9	50.26	
	24.78	2.83	22.25	12.8	62.66	
	5.23	44.34	27.86	0.75	78.18	
	16.1	28.09	6.73	48.03	98.95	
	14.11	29.06	21.03	35.67	99.87	
	15.33	26.04	2.46	2.56	46.39	
	0	0	0	5.22	5.22	
	59.58	5.63	27.47	0.25	92.93	
	46.09	9.49	20.67	22.92	99.17	
	16.81	52.24	18.66	11.24	98.95	
	23.1	2.73	30.81	24.28	80.92	
48 h	19.03	5.03	42.72	7.98	74.76	
	14.3	23.03	48.71	1.43	87.47	
	41.62	6.23	16.35	34.27	98.47	
	13.18	24.83	29.11	18.68	85.8	
	8.23	43.13	2.89	44.01	98.26	
	0	36.35	10.27	9.17	55.79	
	6.64	0	0	14.49	21.13	
	0	0	0	2.16	2.16	
	5.97	53.54	4.49	18.59	82.59	
	40.88	7.9	12.83	36.37	97.98	
	9.8	4.13	2.47	20.4	36.8	
	0.91	0	13.63	0	14.54	
	17.05	0	16.28	5.86	39.19	
	0	1.23	0	0	1.23	
	21.57	7.93	42.71	27.07	99.28	
	60.16	2.63	34.68	2.36	99.83	
	0	17.04	0.35	49.23	66.62	
	10.61	7.92	6.39	59.35	84.27	

Supplemental Table S2: Statistical details (Kruskal-Wallis tests) on the comparison of odor from *Pinus sylvestris* subjected to different treatments: (i) without *Diprion pini* egg deposition, involving exposure to either *D. pini* pheromones (P) or to hexane (H) or were left untreated (U) and (ii) with sawfly egg deposition (E) that followed the same treatments as described for (i). Headspace samples were collected repeatedly from each tree at four different time intervals after the end of pine treatments without egg deposition (0, 24, 48, 72 h) and after the pine treatments with egg deposition (24, 48, 72, 96 h). The emission rates (means ± SE, ng x h^{-1} x $tree^{-1}$) of pine key terpenoids that are known to attract the egg parasitoid *Closterocerus ruforum* are given as well as the *p* values of the statistical comparisons.

Compound	Time point (h)	Without egg deposition				Time point (h)	With egg deposition			
		Untreated (U)	Hexane (H)	Pheromone (P)	Group comparison (U vs H vs P) P-values		Untreated + Oviposition (U+E)	Hexane + Oviposition (H+E)	Pheromone + Oviposition (P+E)	Group comparison (U+E vs H+E vs P+E) P-values
(*E*)-β-ocimene	**0**	0.48 ± 0.10	0.56 ± 0.20	0.20 ± 0.06	0.14	**24**	0.48 ± 0.12	0.62 ± 0.07	1.15 ± 0.36	0.35
	24	0.51 ± 0.07	0.41 ± 0.09	0.22 ± 0.08	0.19	**48**	0.79 ± 0.33	0.82 ± 0.17	0.61 ± 0.19	0.94
	48	0.35 ± 0.04	0.46 ± 0.13	0.23 ± 0.11	0.30	**72**	0.49 ± 0.15	0.60 ± 0.13	0.6 ± 0.13	0.89
	72	0.37 ± 0.08	0.31 ± 0.04	0.17 ± 0.06	0.19	**96**	0.74 ± 0.21	0.73 ± 0.18	1.17 ± 0.45	0.75
(*Z*)-β-ocimene	**0**	0.16 ± 0.03	0.25 ± 0.06	0.17 ± 0.02	0.45	**24**	0.97 ± 0.44	0.69 ± 0.36	0.95 ± 0.26	0.29
	24	0.18 ± 0.03	0.18 ± 0.02	0.13 ± 0.01	0.29	**48**	0.84 ± 0.32	0.97 ± 0.54	0.62 ± 0.29	0.61
	48	0.14 ± 0.02	0.18 ± 0.03	0.12 ± 0.02	0.40	**72**	0.84 ± 0.32	0.82 ± 0.41	0.32 ± 0.07	0.71
	72	0.13 ± 0.02	0.18 ± 0.02	0.13 ± 0.01	0.22	**96**	1.26 ± 0.64	0.92 ± 0.52	0.59 ± 0.20	0.53
β-caryophyllene	**0**	0.94 ± 0.47	3.67 ± 2.26	1.54 ± 1.23	0.76	**24**	2.39 ± 1.02	1.39 ± 0.52	2.68 ± 1.34	0.85
	24	0.73 ± 0.25	1.65 ± 1.00	2.19 ± 1.96	0.69	**48**	2.55 ± 0.96	1.98 ± 0.73	1.29 ± 0.60	0.83
	48	0.74 ± 0.33	3.44 ± 2.64	3.23 ± 2.73	0.99	**72**	2.48 ± 0.99	0.90 ± 0.31	1.24 ± 0.50	0.53
	72	0.81 ± 0.35	2.22 ± 1.66	1.54 ± 0.94	>0.99	**96**	2.35 ± 0.86	1.62 ± 0.39	0.97 ± 0.31	0.64
α-humulene	**0**	0.24 ± 0.10	0.78 ± 0.42	0.39 ± 0.26	0.74	**24**	0.48 ± 0.20	0.39 ± 0.08	0.63 ± 0.20	0.61
	24	0.18 ± 0.05	0.34 ± 0.16	0.47 ± 0.36	0.78	**48**	0.47 ± 0.17	0.48 ± 0.15	0.32 ± 0.10	0.88
	48	0.23 ± 0.08	0.62 ± 0.43	0.54 ± 0.40	1.00	**72**	0.39 ± 0.17	0.26 ± 0.07	0.24 ± 0.08	0.91
	72	0.21 ± 0.07	0.38 ± 0.23	0.31 ± 0.15	0.92	**96**	0.36 ± 0.15	0.31 ± 0.07	0.16 ± 0.02	0.70
(*E*)-β-farnesene	**0**	1.69 ± 0.28	1.25 ± 0.23	1.31 ± 0.28	0.56	**24**	3.31 ± 0.48	5.05 ± 1.35	7.80 ± 2.40	0.31
	24	1.77 ± 0.34	0.97 ± 0.19	1.26 ± 0.26	0.18	**48**	3.48 ± 0.42	5.50 ± 1.54	7.62 ± 1.80	0.11
	48	1.49 ± 0.29	1.11 ± 0.26	1.25 ± 0.24	0.61	**72**	3.69 ± 0.80	5.55 ± 1.73	6.57 ± 1.81	0.63
	72	1.67 ± 0.37	1.04 ± 0.25	1.21 ± 0.27	0.37	**96**	3.36 ± 0.58	4.33 ± 1.19	5.44 ± 1.63	0.77
β-phellandrene	**0**	2.5 ± 0.69	1.65 ± 0.55	1.82 ± 0.43	0.68	**24**	2.53 ± 0.55	3.69 ± 0.81	4.09 ± 1.26	0.47
	24	3.91 ± 2.10	1.14 ± 0.36	1.84 ± 0.62	0.36	**48**	2.99 ± 0.80	3.06 ± 0.61	2.21 ± 0.46	0.76
	48	2.67 ± 1.02	1.40 ± 0.50	1.57 ± 0.52	0.79	**72**	2.85 ± 0.56	4.63 ± 1.63	1.89 ± 0.39	0.50
	72	2.94 ± 1.28	1.65 ± 0.36	1.27 ± 0.46	0.67	**96**	2.81 ± 0.67	4.45 ± 1.90	1.93 ± 0.09	0.72

Supplemental Table S3: Statistical details (Mann-Whitney *U* test) on the analysis of the impact of *Diprion pini* egg deposition on the emission rates of *Pinus sylvestris* key terpenoids that are known to attract the egg parasitoid *Closterocerus ruforum*. Statistical comparison of the emission rates that were recorded by GC-MS analysis of odor from untreated pine (U) and pine with sawfly egg deposition (E) (compare Table S2: U versus U+E). Headspace samples were collected repeatedly from each tree at four different time intervals after the end of the egg deposition period (24, 48, 72, 96 h). Red: *p* values < 0.05.

Compound	Time point (h)	P-values
(*E*)-β-ocimene	24	0.94
	48	0.75
	72	0.94
	96	0.28
(*Z*)-β-ocimene	24	0.01
	48	0.03
	72	0.02
	96	0.02
β-caryophyllene	24	0.28
	48	0.05
	72	0.09
	96	0.23
α-humulene	24	0.34
	48	0.10
	72	0.62
	96	0.94
(*E*)-β-farnesene	24	0.01
	48	0.01
	72	0.04
	96	0.12
β-phellandrene	24	0.87
	48	0.94
	72	0.43
	96	0.94

Chapter 4: Needle Age-Dependent Defence of Scots Pine Against Insect Herbivory

For the journal: *Tree Physiology*

Needle Age-Dependent Defence of Scots Pine Against Insect Herbivory

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Running head:

Age-dependent defence of conifer foliage

Abstract

Plant defence responses to herbivory vary with foliage age, which in angiosperms lasts a few months, but may be several years in conifers. While age-dependent leaf responses of angiosperms to insect herbivory are well studied, much less is known about anti-herbivore responses of conifer needles that differ in age by a year or more. Similarly, insect responses to conifer foliage that differs in age by years have rarely been studied. Here we used Scots pine (*Pinus sylvestris*) and the herbivorous sawfly *Diprion pini* to elucidate (i) the responses of this herbivore to pine needles of different age and (ii) needle age-dependent differences in two anti-herbivore plant defence traits, rarely studied in interactions of conifers and phyllophagous insects: phytohormones and proteinase inhibitor (PI) activity.

The sawfly *D. pini* preferred old (previous-year) pine needles over young (current-year) ones for oviposition and feeding by late larval instars. The insect benefitted from these preferences by higher egg survival rates on old needles and more larval weight when feeding upon old needles.

Scots pine needles showed needle-age dependent differences in their phytohormonal responses to *D. pini* larval feeding. Feeding-induced concentrations of salicylic acid and abscisic acid were higher in young than old needles. No such age-dependency was detected for herbivory-induced changes in levels of jasmonic acid (JA), in contrast to known, age-dependent JA responses to damage in angiosperms. Contrary to angiosperms, PI activities of pine were not induced by sawfly feeding, neither in young nor old needles. However, old needles showed constitutively significantly lower PI activities, which are discussed with respect to the benefits that *D. pini* gains when preferring old needles. Our results highlight the developmental plasticity of defence traits of conifer foliage as well as the adaptation of a pine herbivore specialist to these defences.

Keywords: gymnosperms, plasticity, sawfly, foliage, proteinase inhibitor, phytohormone.

Introduction

Evergreen conifers show the remarkable ability to retain their needle-shaped leaves year-round (Bartels 2021). The evergreen appearance of conifers is due to the excellent adaptation of these trees to cold (Chang et al. 2020) that allows retaining young needles on the tree throughout the year. Detachment of needles occurs only a year or several years after needle flushing. Thus, leaves of evergreen conifers show a much broader age spectrum than deciduous trees.

The relationship between leaf age and plant defences against herbivorous insects has been studied especially in deciduous, angiosperm plants (Feeny 1970, Coley 1980, Gershenzon and Ullah 2022). These studies focused on comparisons of young, flushing leaves and mature leaves, which fully developed within the same year (Barton et al. 2019). In some evergreen conifers, the impact of leaf (needle) age on morphological and chemical traits has been studied on a longer time scale by comparing current-year and previous-year needles. Like the leaf traits of angiosperms, these traits are known to change in dependence of needle age, where younger needles show e.g. higher nitrogen concentrations but lower concentrations of defensive secondary plant compounds like phenolics than older ones (Hatcher 1990, Moreau et al., 2003, McCall and Fordyce 2010).

So far, only limited knowledge is available on how those insects, which use the foliage of conifers for egg deposition and larval feeding, cope with the varying quality of current-year and previous-year conifer needles. A small family of folivorous insects that may occur in high abundances and heavily damage forests are diprionid sawflies (Hymenoptera: Diprionidae) (Schwenke 1982, Davis et al. 2023). Several studies have investigated the impact of needle toughness and concentrations of terpenoids, phenolics, minerals, nitrogen or protein on the performance and preferences of diprionid sawflies (Niemala et al. 1982, Larsson et al. 1986, Björkman and Gref 1993, Lyytikäinen 1994, Pasquier-Barre et al. 2001, Moreau et al. 2003, Giertych et al. 2007). Results of the studies on the impact of needle age on diprionid performance range from toxicity of young pine foliage for *Diprion pini* (L.) (Geri et al. 1988, CABI 2022) to larval-instar dependent suitability of different balsam fir needle ages for *Neodiprion abietis* (Harris) (Moreau et al. 2003).

Although phytohormone levels are well known to differ in juvenile and mature plant tissue (Wasternack and Hause 2013, Berens et al. 2019, Rankenberg et al. 2021), it remains unknown how phytohormone concentrations involved in plant anti-herbivore defences (Walling 2000, Wu and Baldwin 2010, Erb et al. 2012) differ between current- and previous-year conifer needles damaged by sawfly larval feeding. Furthermore, no study of conifer – sawfly interactions investigated yet the role of proteinaceous plant defensive compounds such as proteinase inhibitors (PI). These compounds are known to be wound-inducible in many plant species (Green and Ryan 1972, Tscharntke et al. 2001, Fabisch et al. 2019, Zhu-Salzman and Zeng 2015) and to impair the digestibility of plant proteins (Gatehouse 2011, Fürstenberg-Hägg et al. 2013). In deciduous angiosperms, wound-induced PI activity was found to be dependent on the plant's age and to be higher in young than mature plants (Wolfson and Murdock 1990, Van Dam et al. 2001).

We addressed the above-mentioned gaps in knowledge (i) from the insect's perspective by studying how diprionids are responding to needles of different age and (ii) from the plant's perspective by analysing how conifer needles of different age respond to insect herbivory. For our study, we used Scots pine (*Pinus sylvestris* L.) and the pine sawfly *D. pini*. This sawfly species is a major pest whose females deposit their eggs in a row on pine needles. The hatching larvae gregariously feed on European Scots pines, their primary host (Auger and Géri 1993, Herz and Heitland 1999). Needles of Scots pine usually become two to four years old until they are shed from the tree (Pensa and Jalkanen 2005, Nárovec and Nárovcová 2012). The degree of pine

defoliation by *D. pini* larvae can range from moderate to severe, resulting in significant economic repercussions caused by substantial growth loss and increased susceptibility to secondary pests (Herz and Heitland 1999, Lyytikäinen-Saarenmaa and Tomppo 2002).

In detail, we first addressed the question of how *D. pini* copes with the different quality of current- and previous-year pine needles. Thus, we first studied by laboratory assays the preferences and performance of *D. pini* exposed to current- and previous-year needles of the young Scots pine trees used here. To elucidate how pine needles of different age respond to *D. pini* infestation, we measured the concentrations of the phytohormones jasmonic acid (JA), jasmonic acid isoleucine (JA-Ile), salicylic acid (SA), and abscisic acid (ABA) in control pine needles and feeding-damaged ones of different age. Finally, we also determined the proteinase inhibitor activity in control and feeding-damaged current-year and previous-year pine needles. Hence, these plant analyses aim to gain insight into needle-age dependent phytohormonal responses of Scots pine to *D. pini* infestation and into the possible role of protein-based defence against this pine defoliating insect species.

Materials and methods

Plants and insects

Three-year-old Scots pine trees (*P. sylvestris* L, 30-40 cm in height and 2-4 cm in stem diameter) of a single genotype were purchased from Baumschule Stackelitz GmbH and Co. KG, Stackelitz, Germany. Prior to the experiments, trees were individually potted into new soil (peat and clay, N = 340 $mg \cdot L^{-1}$, P_2O_5 = 260 $mg \cdot L^{-1}$, K_2O = 330 $mg \cdot L^{-1}$) and grown in a greenhouse under long-day conditions (18 hours light, 6 hours dark, with an average temperature of 20°C). The trees selected for the experiments had both young (current-year) and old (previous-year) needles at the same time. The old needles were predominantly needles from the previous year (one-year-old) and only some from a year earlier (two-year-old).

Diprion pini were reared in a climate chamber (18 hours light, 6 hours dark, 20°C, 70% relative humidity) following Eichhorn's (1976) protocol. Scots pine twigs, collected from the Grunewald Forest in the southwest of Berlin, were placed in vases filled with tap water and enclosed in transparent Plexiglas® cylinders (15.08 L) with gauze lids on top. Adult *D. pini* were added for mating and egg deposition. Eggs were laid on pine needles in a row of usually 10-15 eggs per row. In our rearing, larvae began hatching from the eggs 12 to 14 days after egg deposition and started feeding on the needles immediately. Throughout their development, larvae were provided with Scots pine twigs as required, and vessel water was changed every second day. The larvae underwent five instars (males) or six instars (females) before pupation (Hundacker et al. 2024). Upon pupation, cocoons were collected and stored at 4-6°C. Under natural conditions, the pupal phase lasts about two to three weeks or spans the winter, depending on the season of pupation.

Experiments: General

All experiments were conducted in a climate chamber (18 hours light, 6 hours dark, 20°C, 70% relative humidity, 280 µmol photons m^{-2} s^{-1}).

At least three days prior to the experiments, trees were transferred from the greenhouse to the climate chamber for acclimation. For all experiments, a pine tree with both young and old needles was placed in a transparent Plexiglas® cylinder (14.1 L, 80 cm high, 15 cm diameter). The cylinder was equipped with a gauze lid and maintained under constant airflow (250 mL min^{-1}) through an inlet at the bottom of the cylinder.

About four to five days prior to the experiments, *D. pini* cocoons that had been kept cool (4-6°C) were transferred to the climate chamber in which they had spent their juvenile development. Adults emerged about two to three days after transfer.

Oviposition preference and egg survival

To assess whether *D. pini* females exhibit an oviposition preference for young or old pine needles, two sawfly females and two males (two to three days old) were introduced into a transparent Plexiglas® cylinder containing a pine tree with both young and old needles. The females were allowed to oviposit for a three-day period. After this time, the females were removed, and the number of eggs on each needle category was recorded. This assay was conducted four times, with five trees per trial, resulting in a total of n = 20 replicates.

For determination of egg survival, a similar setup was used as for the oviposition preference assay. However, during the oviposition period, only one needle category (either young or old) was exposed for oviposition, while the other needles were enclosed in a mesh bag to prevent contact. The numbers of eggs and of hatching larvae were recorded for each tree. The egg survival rate was determined by calculating the ratio of the total number of eggs laid to the number of larvae that successfully hatched on each needle type per tree. For each needle category, we performed seven independent replicates (n = 7).

Larval preference and performance assay

To assess whether *D. pini* larvae show a feeding preference for young or old pine needles, a pine tree was placed in a transparent Plexiglas® cylinder of the same type as used for the oviposition assays. Young larvae (two to three days old) were collected from a separate setup, and ten individuals were carefully placed on the stem section between the young and old needles of pine trees. Thus, larvae had access to both needle categories. The position of the larvae feeding on specific needle categories was recorded after three, six, and 12 days. After 12 days, the trees began to run low of any of the needle category. We conducted n = 5 replicates.

To determine the performance of larvae, we recorded the weight of larvae feeding on young or old needles. We focused on larval weight as a proxy for performance because it reflects the extent of successful nutrient uptake, utilization and/or conversion of ingested food. We selected larvae of uniform age (two to three days old) and size at the start of the experiment, with all individuals being in the early second instar stage. This approach intended to minimize variability in the initial larval weight and developmental stage. Thus, significant differences in weight of larvae that occurred over the experimental period of 12 days are considered to reflect differences in a performance trait due to feeding on young or old needles. For this performance assay, a similar experimental setup was used as described above for the feeding preference assay. Ten larvae were placed on either young or old pine needles of a pine tree kept in a cylinder. The alternate needle type was enclosed in a mesh bag during the feeding period to prevent larval movements between young and old needles. Larvae were weighed after three, six, and 12 days. We had three trees for each needle category and feeding period. The total number of larvae per feeding period and tree category (access to either young or old needles) varied due to mortality or escape (n = 24-27).

Plant treatments and sampling for chemical analysis of needles

For determination of phytohormone concentrations and proteinase inhibitor (PI) activity in response to *D. pini* larval feeding on either young or old pine needles, trees were subjected to controlled larval feeding treatments. During treatments only one needle age category was exposed at the same time, while the other needle type was enclosed in a mesh bag to prevent

interaction. Again, we kept one tree per cylinder. For larval feeding, ten larvae (two to three days old) were placed on either young or old needles. They were allowed to feed for 24, 48, or 72 hours. Feeding-damaged needles were then collected for analysis. For untreated controls respective to these treatments, a needle age class (young or old) was enclosed, but no larvae were placed on the other needle class.

The number of replicates per treatment varied between n = 5 and 13. This variability in replicates was due to difficulties in collecting sufficient same-age larvae for feeding trials made on the same day, though we always aimed for the maximum number whenever feasible.

For phytohormone analyses, we sampled all locally treated needle pairs that were fed upon. For determination of needle PI activity, systemic needle pairs (adjacent to the treated ones) were collected. Furthermore, needles were collected from untreated control trees at the same time points as from the treated trees. The collected needle material was placed into pergamin bags (65 x 90 mm, Carl Roth GmbH and Co. KG, Karlsruhe, Germany) and immediately frozen in liquid nitrogen. After the initial freezing, the samples were stored at -70 °C. Once all harvesting was complete, the individual frozen samples were ground into a fine powder using liquid nitrogen and returned to -70°C for later phytohormone and protein extraction.

Phytohormone extraction and chemical analysis

Phytohormone concentrations (JA, JA-Ile, SA, ABA) were determined from each needle sample type (i.e, young and old needles, locally feeding-damaged for 24, 48, and 72 hours, and simultaneously sampled young and old untreated controls). Prior to further processing, we determined the weight of each individual sample (ground, powdery samples stored at -70°C) for later exact normalization to needle weight. Overall, sample weights ranged between 95 to 105 mg. The phytohormone extraction was performed following the protocol of Wang et al. (2007) with minor adjustments.

Each sample was transferred to 2 mL tubes containing three to four zirconium beads (Zirconox®, 2.8 – 3.2 mm, Mühlmeier GmbH and Co. KG, Bärnau, Germany). In the following step, 1 mL ethyl acetate containing 2 µL of an internal standard (ISTD) mix of deuterated phytohormones (10 ng μL^{-1} of each D_4-salicylic acid, D_6-abscisic acid, D_6-jasmonyl isoleucine and 30.2 ng μL^{-1} of D_6-jasmonic acid) was added to each sample tube. The deuterated phytohormones were purchased from HPC Standards GmbH, Borsdorf, Germany. We could process a maximum of 22 samples at the same time, therefore, we had to perform the extraction several times to cover all the samples, and for each set (batch) we also prepared blanks. Two blanks containing only 1 mL ethyl acetate, the ISTD mix and grinding beads were also prepared at the same time for each batch. Samples were then subjected to high-speed homogenization (Precellys Evolution Homogenizer, Bertin Technologies SAS, Montigny-le-Bretonneux, France) for two 30-sec cycles (5,000 rpm) with a 5-sec break between cycles. Following homogenization, samples were centrifuged at 10,000 rpm for 10 min at 4°C, and the resulting supernatant was collected. The extraction was then repeated with fresh 1 mL ethyl acetate without internal standards, and the supernatants from both extractions were combined. This combined extract was concentrated using an evaporator at room temperature until it reached a honey-like consistency. Finally, the concentrated extract was re-dissolved in a solution of 70% methanol with 0.1% formic acid and vortexed for 10 min at room temperature. An additional blank containing only the re-dissolution solution (70% methanol with 0.1% formic acid) was also prepared at the same time for each batch of samples. The re-dissolved samples were then centrifuged at 10,000 g for 10 min at 4°C, and the resulting supernatants were transferred to specific vials (200 µL) with inlays and springs.

Chemical analysis was made using UPLC-ESI-MS/MS (Q-ToF-ESI) on a Synapt G2-S HDMS system (Waters Corporation). A sample volume of 7 µL was injected. Components were separated on a C_{18} column (Acquity UPLC BEH C18, Ø 2.1 mm x 50 mm, particle size 1.7 µm;

Waters Corporation, Milford, MA, USA). The mobile phase was a mixture of water (eluent A) and methanol (eluent B), each containing 0.1% formic acid (v/v). Samples were eluted at a constant flow rate of 250 µL min^{-1} at 30°C in an 8-min-lasting gradient mode starting with 30% eluent B, increasing the percentage of eluent B at minute 4.5 to 90%, which was kept until the end of minute 8. Compound detection was made by tandem mass spectrometry scanning the mass spectrum of compounds between 50 – 600 *m/z*. Mass spectra of the separated compounds were obtained by electrospray ionization with defined parameters including capillary voltage (2.5 kV), nebulizer pressure (6 bar), desolvation gas flow rate (500 L h^{-1}), source temperature (80°C), and desolvation temperature (150°C, N_2 as desolvation gas). The phytohormones were identified based on their parent ($[M-H]^-$) and daughter ions. These identifications were confirmed by co-elution of the corresponding deuterated internal standards (ISTD). Specific parent/daughter ion pairs were used to identify SA (*m/z* 137 and 93), ABA (*m/z* 263 and 153), JA (*m/z* 209 and 59), and JA-Ile (*m/z* 322 and 130), along with their deuterated counterparts, i.e. D_4- SA (*m/z* 141 and 97), D_6- ABA (*m/z* 269 and 159), D_6-JA (*m/z* 215 and 59), and D_6- JA-Ile (*m/z* 328 and 130).

Phytohormones were quantified by calculating the ratio of the daughter ion peak area of the specific target to the daughter ion peak area of the corresponding ISTD. MassLynx software (version 4.1, Waters Corporation, Milford, MA, USA) performed these calculations. Finally, concentrations were normalised based on the exact amount measured of the ground needle samples.

Protein extraction and PI activity measurement

Proteinase inhibitor (PI) activities were determined from each needle sample type (i.e, young and old needles adjacent to 24-, 48-, and 72-hour-feeding-damaged needles, and simultaneously sampled young and old needles from untreated controls). To measure PI activity of pine needles, protein extraction from pine needles was made using the Pierce Plant Protein Extraction Kit (Thermo Fisher Scientific Inc, Waltham, MA, USA). The extraction buffer (native lysis) of this kit was diluted (1:2 dilution with ddH_2O). For each sample, 150 µL of the diluted buffer were added to 50 mg ground needle tissue, which had been stored at -70°C prior to precise weighing in a 2 mL microcentrifuge tube (neoLab Migge GmbH, Heidelberg, Germany). To obtain a proper mixture of needle tissue and buffer, the tubes were gently shaken upside down four to five times. Thereafter, the mixture was placed on a shaker (150 rpm) for 15 min at room temperature (Rotamax 120, Heidolph Instruments GmbH and Co. KG, Schwabach, Germany). Finally, the samples were centrifuged at 10,000 x g for 10 min at room temperature. The supernatant was carefully transferred into 2 mL Eppendorf tubes. This extract was used to perform an enzyme assay for determination of PI activity.

The activity of PI was assayed spectrophotometrically (410 nm) by measuring the residual trypsin activity in a trypsin-spiked sample of pine protein extract, hypothesised to exhibit trypsin inhibitor activity. To do so, a modified method described by Kakade et al. (1974) was used to determine the PI activity of pine needles. This standard method has been applied for the analysis of trypsin inhibitor activity in many different plant species (e.g. Momonoki et al. 2002, Gandreddi et al. 2015). BAPNA (N_α-benzoyl-L-arginine *p*-nitroanilide hydrochloride) was used as substrate, and bovine trypsin as standard protease enzyme. The reaction of BAPNA and bovine trypsin results in cleavage of yellow *p*-nitroaniline from BAPNA; the concentration of yellow *p*-nitroaniline serves as a proxy for trypsin activity. Increasing *p*-nitroaniline formation correlates with increasing trypsin activity and optical density. High PI activity reduces trypsin activity and optical density.

To start the measurement, a trypsin solution (1 mg of bovine trypsin in 5 mL of 50 mM Tris-HCl with 30 mM CaCl2; pH 7.4) and a BAPNA solution (5 mg BAPNA dissolved in 1 mL DMSO) were prepared. For the reaction mixture, 35 µL needle protein extract and 20 µL trypsin solution were combined and incubated at 37°C for 15 min in a thermomixer at 700 rpm (Thermomixer comfort,

Eppendorf SE, Hamburg, Germany). Then, 40 µL of BAPNA solution were added, and the mixture was incubated again at 37°C for 20 min in a thermomixer at 900 rpm. Thereafter, the reaction was rapidly terminated by adding 500 µL of 10% glacial acetic acid.

To correct for absorbance caused by needle chlorophyll present in the protein extract and to measure baseline absorbance, a blank was prepared for each sample. This blank consisted of the same protein extract as the sample, but acetic acid was added prior to BAPNA to prevent reaction. The "baseline absorbance" of this blank was subtracted from the absorbance of the BAPNA- and trypsin-treated needle protein extract. Additionally, a positive control representing maximum trypsin activity (100 %) was used by replacing the protein extract by the extraction buffer only.

For PI activity measurement, 250 µL of each reaction mixture with respective controls were placed separately in a 96-well plate. Absorbance was measured at 410 nm using a spectrophotometer (Infinite® 200 M Nano+, Tecan Group AG; software: i-control 2.0). To calculate and normalise the PI activity, the absorbance values of the test samples were first corrected using respective blank samples. After blank correction, the absorbance of each sample was normalised to the corresponding positive control on the same 96-well plate. This normalisation allowed for the comparison of PI activity between plates, even when slight variations in positive control absorbance occurred. The percentage of trypsin activity was calculated using the following formula:

$$\text{Trypsin Activity (\%)} = \left(\frac{100}{\text{Positive control absorbance}}\right) \text{x Sample absorbance}$$

Statistical analysis

Statistical analysis was performed using GraphPad Prism (V 10.2.1) and R (version 4.3.2) with R Studio (version 2023.12.0+369) for detailed data analysis (R Core Team, 2023). Data distribution was assessed using the Shapiro-Wilk method for normality check, followed by appropriate statistical tests. p-values greater than 0.05 were considered not significant (ns), while p-values below 0.05 were considered significant.

The non-normally distributed data obtained by the oviposition preference assay providing a choice between young and old needles were statistically analysed by the Wilcoxon matched-pairs signed rank test. The normally distributed egg survival data were statistically evaluated by an unpaired *t*-test.

For statistical evaluation of the larval feeding preference assay, we calculated the percentage of larvae on young and old needles, respectively (100% = sum of larvae detected on young and old needles). The resulting data were analysed by Wilcoxon matched-pairs signed rank test.

We used the Mann-Whitney *U* test for the analysis of the non-normally distributed data of the weight data of larvae feeding on young and old needles and for the phytohormone concentrations in young and old needles. We again used the Mann-Whitney *U* test for statistically comparing proteinase inhibitor activities of (i) untreated, young control needles with those of untreated, old control needles, (ii) young, feeding-damaged needles with those of old, feeding-damaged needles, and (iii) untreated, young (old) needles with those of feeding-damaged, young (old) needles.

Results

Old (previous-year) Scots pine needles are preferred for sawfly oviposition and favour egg survival

To determine whether ovipositing female *D. pini* differentiate between young (current-year) and old (previous-year) Scots pine needles, they were offered pine trees with these types of needles. After an oviposition period of three days, the mean number of eggs laid on old needles was significantly higher than on young needles (Figure 1a).

In a separate experiment, where females could only lay eggs on one needle category (either young or old), the egg survival rate was determined by recording the number of larvae hatching from eggs. Significantly more eggs survived the egg incubation time of at maximum 14 days until larval hatching when they had been laid on old Scots pine needles than on young ones (Figure 1b).

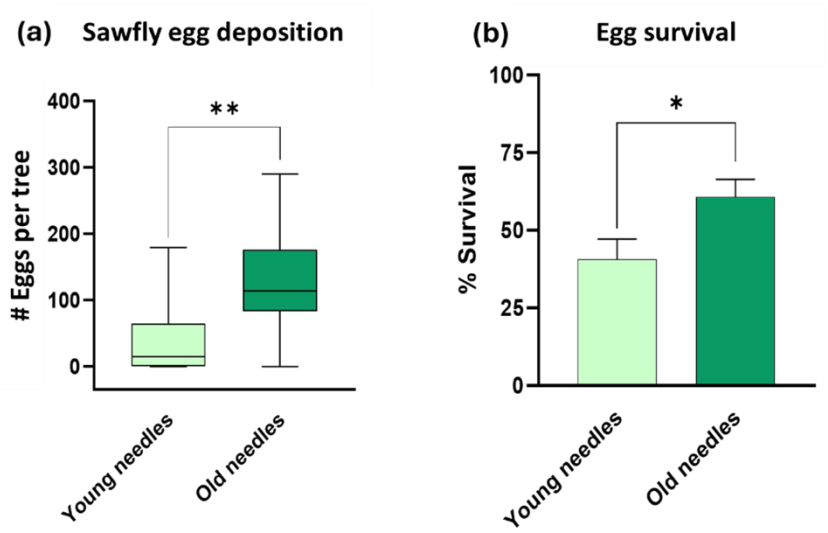

Figure 1. Oviposition and egg survival of *Diprion pini* on young (current-year) and old (previous-year) *Pinus sylvestris* needles. **(a)** Box plots depicting the number of *D. pini* eggs per needle age class. Medians, first and third quartiles, and full range of scores are shown. Trees were presented to *D. pini* females for a period of three days (n = 20 trees, 2 females paired with 2 males per tree). Statistical significance was determined using the Wilcoxon matched-pairs signed rank test (** $p < 0.01$). **(b)** Means (+ SE) depicting the percentage of eggs that survived until larval hatching when eggs were laid on either young or old needles (n = 7 trees/needle age class, 2 females paired with 2 males per tree). Statistical analysis was performed using an unpaired *t*-test (* $p < 0.05$).

A feeding preference shift from young to old needles during larval development is linked with higher larval weight on old needles

To determine whether *D. pini* larvae show a feeding preference for young or old needles of Scots pine, second instar larvae were positioned on a pine tree between young and old needles. Within three days, a tentatively higher proportion of larvae began feeding on young needles than on old ones, although this difference was not statistically significant. This trend persisted until about day six. However, after a feeding period of 12 days, the majority of larvae was found feeding on old needles (Figure 2a).

To further investigate whether the performance of larvae also differs when feeding on young versus old needles, two- to three-day-old larvae were placed on either young or old needles of separate trees and were individually weighed after a three-, six- and 12-day lasting feeding period. No significant differences were found in mean larval weight between those feeding on young needles and those on old needles after a three- and six-day lasting feeding period.

However, after a 12-day feeding period, larvae that had fed on old needles were significantly heavier than those on young needles (Figure 2b).

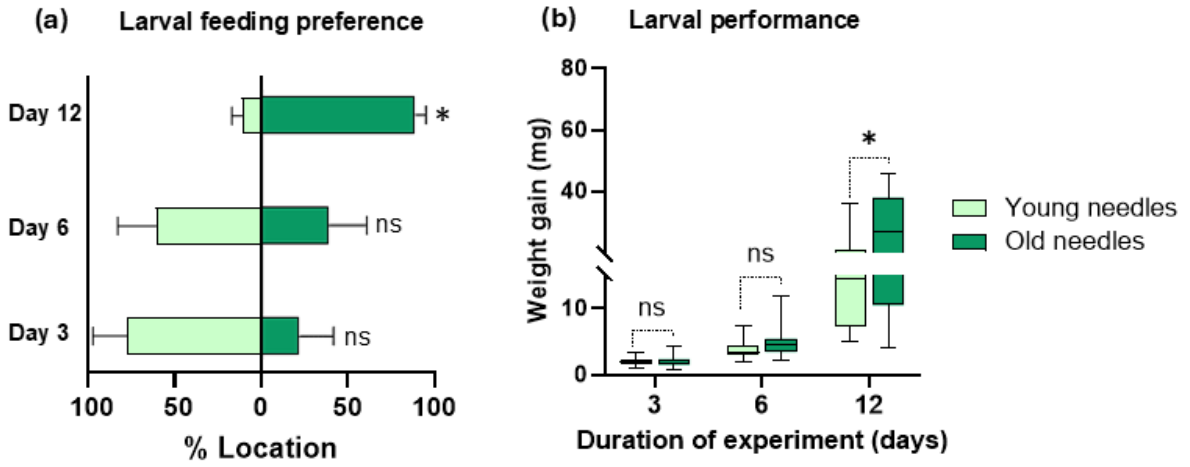

Figure 2. Feeding preferences and performance of *Diprion pini* larvae on *Pinus sylvestris* needles of different age classes. **(a)** Means (+ SE) depicting the percentage of larvae observed on young and old needles after feeding periods of three, six, and 12 days, (100% = sum of larvae on young and old needles; n = 5 trees, 10 larvae per tree in the beginning of the assay). The Wilcoxon matched-pairs signed rank test was used for statistical analysis (ns = not significant; * $p < 0.05$). **(b)** Box plots illustrating larval performance based on weight of larvae when feeding exclusively on young or old needles. Median values, first and third quartiles, and full range of scores are shown. Biological replicates of larvae for each time point on young needles: $n_{day3} = 26$, $n_{day6} = 26$, $n_{day12} = 24$; for old needles: $n_{day3} = 27$, $n_{day6} = 24$, $n_{day12} = 24$). Statistical differences between the two groups were analysed using the Mann-Whitney *U* test (ns = not significant; * $p < 0.05$).

Constitutive and feeding-induced phytohormone concentrations differ in young and old Scots pine needles

We compared the concentrations of the phytohormones JA, JA-Ile, SA, and ABA in young and old pine needles that were either left untreated or exposed to different larval feeding periods. Three types of comparisons were conducted: (i) untreated young versus untreated old control needles (Cy vs Co), (ii) feeding-damaged young versus feeding-damaged old needles (Fy vs Fo), and (iii) untreated young needles versus feeding-damaged young needles (C vs F (y)) as well as untreated old needles versus feeding-damaged old needles (C vs F (o)) (Figure 3, please note bars for comparison (i) and (ii), note depicted p-values for comparison (iii)).

In untreated needles, constitutive concentrations of JA and JA-Ile did not differ between young and old needles, whereas SA and ABA levels were found to be higher in untreated young needles than old ones (Cy vs Co comparisons).

In feeding-damaged samples, JA and JA-Ile concentrations were significantly higher than in untreated control samples (C vs F (y) and C vs F (o)), but there was no significant difference between young and old feeding-damaged needles (Fy vs Fo). In feeding-damaged needles, SA levels were higher in young needles than in old ones after a 24-hour feeding period (Fy vs Fo). When comparing untreated control needles and feeding-damaged ones, SA levels were significantly inducible in old needles only (C vs F (o)). ABA levels were higher in feeding-damaged young than old needles after a 24-hour and 72-hour feeding period (Fy vs Fo) but feeding induced ABA levels both in young and old needles (C vs F (y); and C vs F (o)), regardless of the feeding period.

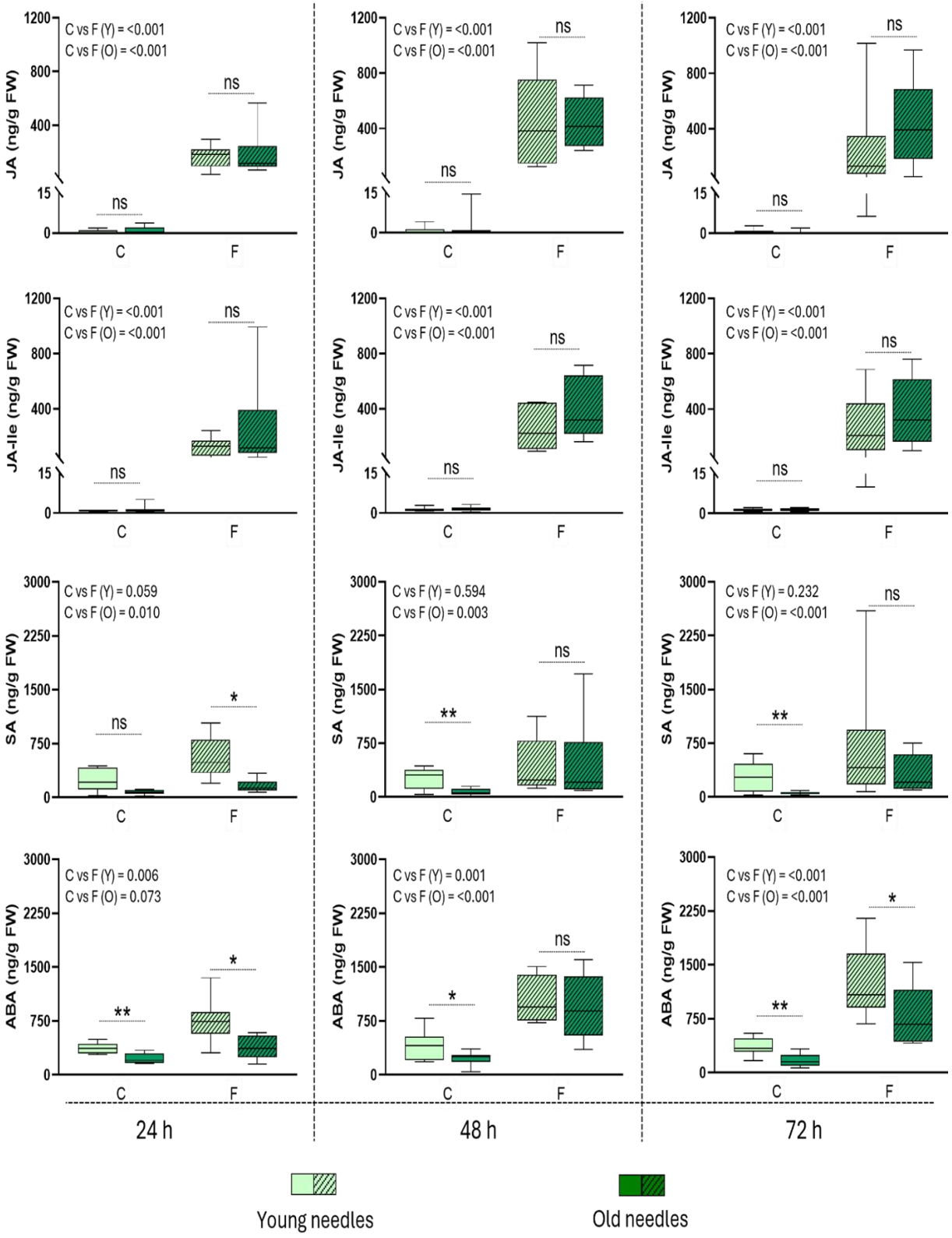

Figure 3. Concentrations of jasmonic acid (JA), jasmonyl isoleucine (JA-Ile), salicylic acid (SA), and abscisic acid (ABA) are measured in ng/g fresh weight (FW) of young (Y) and old (O) *Pinus sylvestris* needles after 24-, 48-, and 72-hour lasting feeding (F) by *Diprion pini* larvae, as well as in untreated controls (C). Locally feeding-damaged needles were analysed. Box plots display the median, first and third quartiles, and the full range of scores. Statistical significance was assessed using the Mann-Whitney *U* test (ns = not significant, * $p < 0.05$, ** $p < 0.01$). Biological replicates for young needles were: 24 h: nC = 7, nF = 8; 48 h: nC = 10, nF = 5; 72 h: nC = 10, nF = 13. For old needles: 24 h: nC = 7, nF = 7; 48 h: nC = 13, nF = 6; 72 h: nC = 10, nF = 8.

Old Scots pine needles show lower proteinase inhibitor (PI) activity than young ones

We measured trypsin activity as an indicator of PI activity in Scots pine needles. Untreated old needles showed significantly higher trypsin activity (correlated with lower PI activity) than young needles. Similarly, feeding-damaged old pine needles showed higher trypsin activity than young ones, regardless of the feeding duration. No significantly feeding-induced changes in trypsin activity were detected in young or old needles (Figure 4).

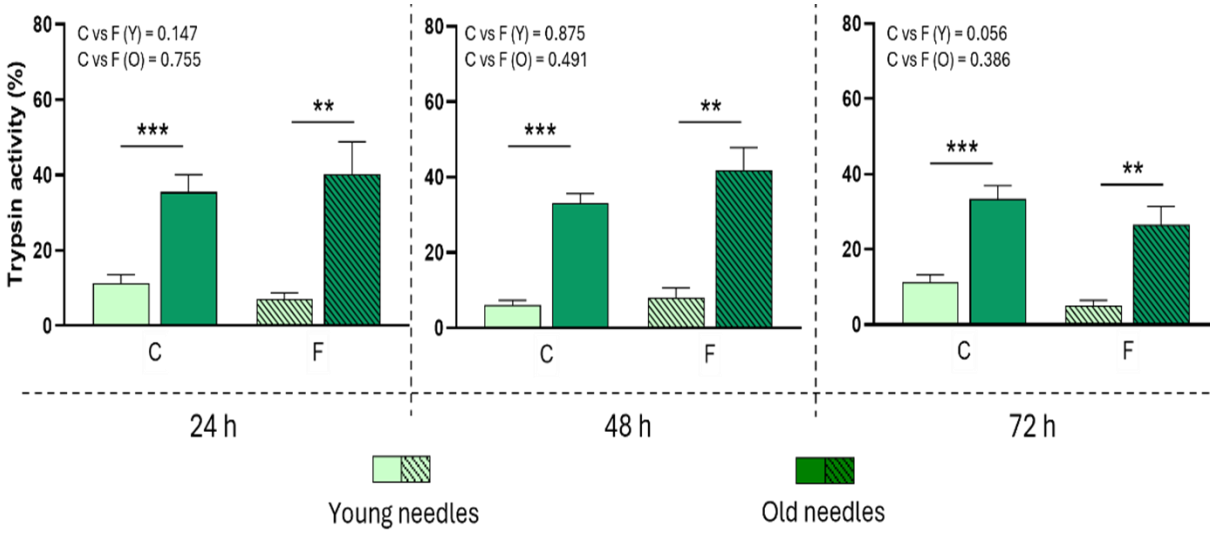

Figure 4. Trypsin activity (mean + SE) in protein extracts from young (Y) and old (O) *Pinus sylvestris* needles after 24-, 48-, and 72-hour lasting feeding (F) by *Diprion pini* larvae, as well as in untreated controls (C). Needles adjacent to the feeding-damaged ones were analysed. Data are presented as the percentage of trypsin activity, calculated by normalizing the absorbance values of each sample to the corresponding positive control absorbance, following correction with respective blanks to account for baseline absorption. Statistical analysis was performed by using the Mann-Whitney *U* test (** $p < 0.01$; *** $p < 0.001$). Biological replicates for young needles were: 24 h: nC = 9, nF = 5; 48 h: nC = 10, nF = 6; 72 h: nC = 10, nF = 6. For old needles: 24 h: nC = 8, nF = 6; 48 h: nC = 8, nF = 6; 72 h: nC = 10, nF = 6.

Discussion

We have used *P. sylvestris* and the herbivorous sawfly *D. pini* to explore how differently young and old foliage of a conifer species respond to herbivory and how in turn the insect is responding to the foliage of different age. Our study revealed that *D. pini* females preferred laying eggs on old, one-year-old pine needles, where egg survival rates were higher than on young needles from the current year. Interestingly, the larvae showed a shift in feeding behaviour during their development. Early instar larvae favoured by trend young needles, while later instars preferred old needles, where they gained more weight. Our phytohormone analyses revealed that young and old needles showed some differences in both constitutive and feeding-inducible responses. Additionally, PI activity was constitutively lower in old needles, but was not induced by larval feeding, neither in young nor old needles.

Below we will discuss the sawfly's behavioural responses to pine needles of different age with special emphasis on the adaptive value of these responses. Furthermore, we will take the plant's perspective and compare the age-dependent conifer foliage responses to herbivory with known anti-herbivore responses of juvenile and mature angiosperm foliage. In addition, we discuss how the measured phytohormone levels might relate to the detected PI activities, and thus to pine defence against sawfly larval herbivory.

Adaptive behavioural responses of a herbivorous insect to pine needles of different age

At a first glance, our finding that larvae benefit from development on old needles by gaining more weight matches the oviposition preference of *D. pini* females for old pine needles. However, in the field, additional or other parameters than larval weight, for example, enemy-free space for eggs and larvae may also favour selection of sawfly oviposition preferences for certain host plant quality (Björkman et al. 1997). Numerous studies have shown a link between insect oviposition preferences and larval performance (Gripenberg et al. 2010, Jones et al. 2019). According to the preference-performance hypothesis (PPH) and "mother knows best" theory, females of herbivorous insects should lay eggs on plants that maximise offspring development and survival (Jaenike 1978, Valladares and Lawton 1991). Females of *D. pini* are known to differentiate between pine species when choosing oviposition sites, but their choices do not always align with optimal conditions for larval performance (Barre et al. 2002). While most studies focus on a linkage between oviposition preference and larval performance, egg survival also needs consideration, particularly because of plant defences induced by egg deposition (Austel et al. 2016, Hilker and Fatouros 2015, 2016, Bertea et al. 2020, Lortzing et al. 2020, Schott et al. 2022). In our study, the oviposition preference of *D. pini* females for old needles matched higher egg survival rates on these needles than on young ones. Future studies need to elucidate which pine needle factors affect *D. pini* egg survival. Bittner et al. (2017) showed that survival rates of *D. pini* eggs on *P. sylvestris* are reduced when hydrogen peroxide concentrations in needles increase. Thus, it will be particularly interesting to figure out whether young pine needles show higher hydrogen peroxide concentrations in response to *D. pini* egg deposition than old ones.

While *D. pini* egg survival and larval weight was higher on old than young pine needles, early instar larvae tended to prefer the young needles, where they would gain less weight if they would keep this preference during their development. Our findings align with the known negative effects of young foliage on early sawfly larvae; feeding upon young pine foliage slows development and reduces weight gain (Auger and Géri 1993). The shift in feeding preferences from young to old needles shown by the *D. pini* larvae during their development suggests that young larvae place different demands for good development than elder ones. A study by Moreau et al. (2003) demonstrated that larvae of *Neodiprion abietis,* a close relative of *D. pini,* performed best on a mixed diet of foliage from various age classes and worst when restricted to young, current-year needles alone. We here determined the weight of larvae feeding on either young or old needles. Therefore, we do not know whether *D. pini* larvae would gain even more weight than those feeding on just old needles (as in our assay) if they would be allowed to feed first on young needles and later on old ones.

Numerous studies have shown that high PI activities in foliage can protect plants from herbivory and negatively affect insect growth and performance (e.g. Hilder et al. 1987, Zhu-Salzman et al. 2008, Zhu-Salzman and Zeng 2015, Singh et al. 2020). In line with these previous findings, *D. pini* larvae gained less weight when they had fed for several days on young pine needles with a constitutively high PI activity. However, we cannot exclude that other foliage parameters than PIs contributed to the reduced weight of larvae that fed upon young needles. For example, high concentrations of resin acids are known to be detrimental to larvae of *Neodiprion sertifer,* especially to the young larvae (Larsson et al. 1986). Low molecular plant secondary compounds are known to shape the effects of plant PIs on insect performance (Steppuhn and Baldwin 2007). Future studies need to elucidate how pine PIs and low molecular secondary needle compounds affect *D. pini* larval performance. With respect to the shift in *D. pini* feeding preferences from young to old needles, special attention should be paid to possibly instar-specific adaptations to the needle's defences (Jongsma and Bolter 1997; Zhu-Salzman and Zeng 2015, Spit et al. 2016).

Phytohormonal responses of young and old foliage to herbivory and their impact on PI activities

In angiosperms and gymnosperms, defences against feeding damage are well known to be linked with a damage-induced increase in JA and JA-Ile concentrations (Chung et al. 2008, Walling 2000, Erb et al. 2012, Pieterse et al. 2012, Yan and Xie 2015, Lortzing and Steppuhn 2016). Levels of these phytohormones increased also in *P. sylvestris* needles damaged by *D. pini* larvae. However, the similar JA and JA-Ile concentrations in young and old feeding-damaged pine needles indicate that needle age does not affect the responses of these phytohormones to damage by sawfly larvae. In contrast, juvenile and mature leaves of *Arabidopsis* are known to differ with respect to their JA and JA-Ile responses to damage; the jasmonate response of mature foliage is attenuated when compared to young foliage (Wasternack and Hause 2013, Mao et al. 2017, Berens et al. 2019, Rankenberg et al. 2021). These findings suggest differences between angiosperms and conifers with respect to their JA- and JA-Ile-regulated responses to feeding damage.

Furthermore, wound-induced increase of JA concentrations in angiosperm plants is well known to be accompanied by an increase in PI activity (e.g. Farmer et al. 1992, Koiwa et al. 1997, Casaretto et al. 2004). However, our data indicate that PI activities of Scots pine were not induced by JA. This indication is based on the finding that (i) wounding by *D. pini* feeding did not induce PI activities and (ii) PI activities were higher in young than old needles, but JA levels did not differ between young and old needles. So far, we cannot exclude that the here detected JA and JA-Ile responses of pine to feeding damage are specific for the *P. sylvestris – D. pini* interaction. We can neither exclude that greater differences in pine needle age than those studied here would reveal also differences in the JA and JA-Ile responses to damage.

While needle age did not affect the JA and JA-Ile concentrations in response to *D. pini* feeding, the feeding-induced SA and ABA concentrations significantly differed between young and old pine needles. Already after a 24-hour lasting feeding period, SA and ABA levels were significantly higher in feeding-damaged young needles than in damaged old ones. Age-dependent changes in concentrations of SA and ABA are also known in angiosperm plants in response to stress. For example, similar to the SA response of young pine needles to feeding damage, also young rosette leaves of *Arabidopsis* show stronger SA responses to biotic stress than old leaves (Zeier 2005). Like in the young pine needles studied here, ABA concentrations of angiosperm leaves are known to be highest in immature leaves and buds (Zeevaart and Boyert 1984, Zeevaart and Creelman 1988). The role of ABA has been intensively studied in angiosperms with respect to plant growth (Humplik et al. 2017, Brookbank et al. 2021). Furthermore, ABA is well known to be involved in numerous defence responses, among them ROS accumulation, defence gene expression (Bari and Jones 2009) and synergism with the JA pathway (Pieterse et al. 2012). In young conifer needles, elevated ABA concentrations were found to enhance stress tolerance and to be crucial for young conifer needles, especially at the crown periphery with high a evaporative demand (Christmann et al. 1995).

The detected higher constitutive SA and ABA levels in young than old Scots pine needles might be important for the higher constitutive PI activity in young needles than in the old ones. This suggestion is supported by findings in some angiosperm plants, in which (genes encoding) PIs were induced by SA (e.g. Raju et al. 2009; Chen et al. 2024). In contrast, SA has been reported to inhibit JA-induced PI activities (e.g. Koiwa et al. 1997). However, as mentioned above, our data indicate that PI activities of Scots pine were not induced by JA. In addition to SA, also ABA might contribute to the high constitutive PI activity of young Scots pine needles. This suggestion is supported by a previous study, which showed that exogenous application of ABA to nonwounded (potato) plants was found to induce PI gene expression to a similar extent as was observed after wounding (Peña-Cortés et al. 1989). Hence, both SA and ABA might be relevant for keeping levels

of PI activity high in young (nonwounded) needles, which might have special needs for PI activity to regulate their needle protein turnover during maturation.

Concluding remarks

In conclusion, our study highlights the relationship between needle age, herbivory, and plant defences in an evergreen conifer species. The oviposition preference of *D. pini* for old needles aligns with the preference-performance hypothesis, where optimal conditions for offspring development guide oviposition choices. Additionally, a notable shift in larval feeding behaviour, with young larvae tentatively favouring young needles and old larvae switching to old needles, suggests that *D. pini* larvae pursue a flexible feeding strategy, which might balance the effects of secondary plant compounds and PI activities on larval performance according to an instar-dependent sensitivity to these plant defences. The hormonal responses of the pine needles to herbivory showed significant increases in JA and JA-Ile in both young and old needles, but SA and ABA levels differed between needle ages, suggesting a more pronounced defensive role of these phytohormones in young needles. Higher constitutive PI activity in young than old needles might contribute to reduced weight of larvae feeding upon young needles. The data suggest a preemptive pine defensive strategy that prioritises high constitutive PI activity to protect young tissues. Diprionidae share a long evolutionary history with pine trees (O'Reilly et al. 2015) and seem to be well adapted to their host plants. Taken together, our results indicate that both pine and the pine sawfly *D. pini* evolved high ontogenetic plasticity of those traits that exert effects on the other species.

The ontogenetic plasticity of needle age-dependent defence against herbivory may be further modulated by the age of the tree. As trees mature, both the chemical and structural composition of their needles could shift, potentially affecting the nutritional value and defence compounds available to herbivores (Nybakken et al. 2018, Lirette and Despland 2021). Younger trees often prioritise growth over defence, while older trees invest more in defensive responses (Lieutier et al. 1993, Barton and Koricheva 2010). In line with this, mature trees often have higher concentrations of defensive compounds in their needles compared to younger trees (Tegischer et al. 2002, Turfan et al. 2018). However, according to the optimal defence theory (ODT, Rhoades and Cates 1976, Stamp 2003, McCall and Fordyce 2010), protection of young leaves should be prioritised because these leaves are considered more valuable to a plant than old ones. The young leaves are considered highly valuable because of their high photosynthetic capacity and expectance of a long life (Harper 1989). Indeed, young, current-year *P. sylvestris* needles were found to show a higher photosynthetic activity than old, 1-year-old needles (Jach and Ceulemans 2000). Hence, future studies need to elucidate whether and how the needle age-dependent changes in defence that we detected here in young Scots pine trees are shaped by the age of the tree.

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Conflict of interest

None declared

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Data availability

All relevant data are presented in the paper.

Authors' contributions

A.R.S. and M.H. conceptualised the study and designed the experiments. A.R.S. carried out the oviposition preference and egg survival experiments. S.K. carried out all experiments with larvae and prepared samples for phytohormone extraction and analysis. L.H. performed the phytohormone extraction. S.K. and A.R.S. conducted protein extraction and measured PI activity. A.R.S. conducted the statistical analyses. A.R.S. wrote the first draft of the manuscript, A.R.S. and M.H. wrote revised versions, and all authors contributed to the final version of the manuscript.

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Chapter 5: General Discussion

My thesis centres around several key aspects of the interactions between pine trees, specifically *Pinus sylvestris*, and the sawfly *Diprion pini*, highlighting the roles of sawfly sex pheromones and needle age in modulating pine defences.

Insect pheromones have widely been studied for their role in insect communication, but our knowledge on their impact on plant responses and subsequent effects on herbivorous insects is surprisingly limited. Exposure of pine trees (*P. sylvestris*) to sawfly (*D. pini*) sex pheromones is known to enhance pine defence against sawfly eggs. In **Chapter 2 and 3** of this thesis, I investigated whether **(a)** sawfly females have developed behavioural counter-adaptations to protect their eggs from increased mortality on pheromone-primed trees. Additionally, I examined whether the priming effect of sawfly sex pheromones on pine's direct defence against eggs extends to **(b)** direct defence against sawfly larvae or **(c)** indirect defence against eggs through enhanced attraction of egg parasitoids. Regarding question **(a)**, my studies showed that sawfly females did not distinguish between the odour from pheromone-exposed and unexposed trees. However, in a no-choice oviposition assay (with contact to the trees), more unexposed than pheromone-exposed trees received eggs. As for question **(b)**, the studies revealed that larval performance and pupation success were unaffected by whether the trees had been exposed to pheromones or not. For question **(c)**, investigation of the effects of pheromone exposure on pine's indirect defence showed that *Closterocerus ruforum*, a key egg parasitoid, did not differentiate between odours from pheromone-exposed and unexposed trees. Additionally, chemical analysis of pine key terpenoid emissions, important for attracting egg parasitoids, revealed that pheromone exposure had no significant impact on the release of these compounds from pine needles, regardless of the presence of eggs. However, consistent with previous studies, sawfly egg deposition induced the emission of (*E*)-β-farnesene, a sesquiterpene crucial for parasitoid attraction.

In **Chapter 4**, I investigated **(a)** how pine needle age influences *D. pini* oviposition and larval feeding preferences, and **(b)** effects of pine needle age on sawfly egg survival and larval development. Additionally, I examined **(c)** the variation in phytohormonal concentrations and proteinase inhibitor (PI) activity in response to feeding sawfly larvae and in dependence of pine needle age. I differentiated between young (current-year) and old (previous-year) pine needles. Regarding questions **(a)** and **(b)**, female sawflies exhibited a strong preference for laying eggs on older needles, where egg survival rates were notably higher. Interestingly, larval feeding preferences varied with instar: early instar larvae tentatively preferred younger needles, while later instars showed a significant preference for older needles alongside increased weight gain. Concerning question **(c)**, young pine needles showed enhanced constitutive defences, exhibiting elevated levels of defence-related phytohormones such as salicylic acid (SA) and abscisic acid (ABA), as well as higher trypsin inhibitor activity. These factors likely contribute to reduced egg survival and diminished larval weight gain on younger needles.

In the general discussion of my thesis, I will first highlight potential mechanisms of pheromone perception in plants (Chapter 5.1). Additionally, the selective nature of pheromone priming in pine will be examined (Chapter 5.2). Furthermore, I will discuss how the pine's responses to pheromones may be integrated into the plant's defence strategies (Chapter 5.2). The discussion will also address how needle age influences anti-herbivore defences in pine trees (Chapter 5.3),

highlighting the differential responses of pine needles of different age and their role in shaping insect behaviour and survival.

5.1 Potential Mechanisms for Pheromone Perception in Plants

In **Chapter 2** of this thesis, we found evidence for a fascinating counter-adaptation by sawfly females to the pheromone-primed direct defences of pine trees. The findings suggest that sawfly females have developed a remarkable strategy of oviposition avoidance in response to the pine trees' pheromone-primed defences. These defences require the ability of pine trees to perceive the volatile pheromones of the sawfly *D. pini*. After pheromone perception, pine trees can obviously prime their defences against sawfly egg deposition as demonstrated previously by Bittner et al (2019). This phenomenon raises the question which mechanisms are involved in recognition of and response to volatile insect pheromones by plants? Understanding how plants perceive and respond to insect pheromones is not only a question of ecological interaction, but also of evolutionary significance. The ability of plants to evolve mechanisms for detecting insect signals and priming their defences suggests a co-evolutionary arms race, where both sawflies and pines are continually adapting and counter-adapting to each other's strategies. Such evolutionary processes shape the interactions between species over time, driving the development of sophisticated defence and avoidance mechanisms.

Given the current lack of direct evidence for receptors of insect pheromones in plants or other modes of detection, only some possibilities can be suggested on how plants perceive volatile insect pheromones. The suggested mechanisms are mainly based on studies of plant perception of stress-induced plant volatiles that allow plant – plant communication (Brosset & Blande, 2021; Loreto & D'Auria, 2022; Kalske & Kessler, 2023).

A plant's response to volatiles requires that the volatile compounds traverse the plant cuticle and reach the cell wall and plasma membrane. Open stomata and passage of volatiles through the cuticle and cell wall allow arrival of volatiles at the plasma membrane (Wang & Erb, 2022). The passage through the cuticle and cell wall is thought to be due to diffusion (Wang & Erb, 2022), but also transfer proteins might facilitate this passage. A recent study has identified non-specific lipid transfer proteins (nsLTPs) that facilitate the emission of floral volatiles. The nsLTPs bind floral volatiles and shuffle them through the cell wall to the outside (Cheng et al., 2004, Missaoui et al., 2021, Liao et al., 2023); these proteins might also help external volatiles traversing the cell wall from outside to the plant inside.

Once arriving at the plasma membrane, several further processing mechanisms have been suggested and will be discussed below. Volatile organic compounds (VOCs) might (i) interact directly with the plant plasma membrane, thus inducing responses by disturbing the architecture of the membrane (Furlan et al., 2020), or (ii) bind to membrane-localised receptors (Wang & Erb, 2022), or (iii) enter the cytosol by diffusion through the plasma membrane for metabolic processing (Matsui, 2016), or (iv) enter the cytosol via a shuttle provided by ATP-binding cassette (ABC)-transporters (Adebesin et al., 2017; Widhalm et al., 2015). Once having reached the intracellular space, (v) volatile compounds may be bound to odorant-binding proteins (OBPs), which might finally deliver the volatiles to intracellular receptors (Giardano et al., 2021; Wang &

Erb, 2022). Thus, employing multiple detection systems for VOCs rather than relying on a single "olfactory" system would be of benefit for plants (Cofer et al., 2018).

(i) Interaction of volatiles with the plasma membrane and disturbance of membrane architecture

Plants might respond to insect pheromones via disturbance of their cell membranes. This would not necessitate the evolution of specific pheromone receptors; instead, it would rely on the plant's inherent ability to detect shifts in membrane integrity, which could trigger downstream defence responses (Zebelo et al., 2012).

Ester-based pheromones, such as (2*S*,3*R*,7*R*)-3,7-dimethyl-2-tridecanyl acetate and propionate produced by sawfly *D. pini* females (Anderbrant et al., 1995; Bergström et al., 1995; Anderbrant et al., 2005), have a somewhat amphiphilic character with their lipophilic dimethyl tridecanyl part and the hydrophilic carboxylic acid part. Such compounds might act like surfactants, which may disturb membrane architecture and fluidity (Furlan et al., 2020), initiating calcium transients, and/or triggering reactive oxygen species (ROS) production. The amphiphilic nature of many pheromones, which have both hydrophilic and hydrophobic regions, enables them to directly interact with the lipid bilayers of plant membranes. Although the impact of insect pheromones on membrane disturbance has not been studied, several studies demonstrated that other VOCs, particularly green leaf volatiles (GLVs), can rapidly alter plasma membrane potential and cytosolic calcium flux in plants that can trigger defence mechanisms and gene expression (Zebelo et al., 2012; Zebelo & Maffei, 2016; Aratani et al., 2023).

(ii) Binding of volatiles to receptors in the plant plasma membrane

The presence of plasma membrane localised receptors of VOCs has been suggested by several authors, but experimental evidence for them is lacking (Wang & Erb, 2022; Kessler et al., 2023; Widhalm et al., 2023). However, several studies investigating the presence of receptor proteins, such as G-protein coupled receptors (GPCRs) (Liu et al., 2007; Gookin et al., 2008; Pandey et al., 2009) and receptor-like kinases (RLKs) (Shiu & Bleecker, 2001; Torii, 2009), suggest that receptors capable of responding to VOCs might be present in the plasma membrane. Furthermore, the rapid responses observed in plants upon exposure to certain volatiles, such as the induction of calcium signalling, suggest the involvement of membrane-bound receptors that can quickly transduce signals.

(iii) and (iv) Passage of volatiles through the plasma membrane

As suggested by Matsui (2016), volatiles that have reached the intercellular space, diffuse through the cell wall and plasma membrane into the cytosol where they will be metabolised. Thus, their cytosolic concentration is lowered, which in turn facilitates – according to the suggestion by Matusi - the uptake into the cytosol, VOCs may also be transported into the cell through protein active systems such as ABC transporters, as suggested by Adebesin et al. (2017). These transporters are integral membrane proteins that utilise ATP to translocate a

wide variety of substrates across cellular membranes (Kang et al., 2011; Do et al., 2018). ABC transporters have a broad substrate range and can transport a variety of molecules, including different classes of compounds (Yazaki, 2006; Kang et al., 2011). ABC transporters may reduce the need for highly specific odorant binding proteins and facilitate flexible response to external signals. ABC transporters can also regulate the intracellular concentration of transported compounds by controlling their uptake and efflux (Linka & Weber, 2010; Zhang et al., 2018). This regulation is essential for maintaining cellular homeostasis and ensuring that signalling molecules are present at appropriate levels to elicit the correct physiological response (Conde et al., 2011; Dahuja et al., 2021). For example, overaccumulation of certain VOCs could be toxic or lead to inappropriate signalling, while insufficient levels might result in a failure to activate necessary defence mechanisms. This would allow for better regulation of signalling molecule levels, which can be crucial for appropriate physiological responses.

(v) Involvement of odorant-binding proteins and intracellular receptors

Odorant-binding proteins (OBPs) are a class of proteins that bind lipophilic volatile organic compounds (VOCs) and transport them to receptor proteins, acting as mediators in the process of chemical signalling. In animals, OBPs "perform a dual function: solubilizing and ferrying volatile pheromones to the olfactory receptors" (Pelosi & Knoll, 2022). In insects, many genes encoding OBPs have been identified (Rihani et al., 2021). OBPs have been hypothesised to exist in plants as well (Loreto & D'Auria, 2022).

In plants, OBPs have been suggested to be involved in binding VOCs emitted by neighbouring plants, aiding in plant – plant communication and defence (Giordano et al., 2021; Loreto & D'Auria, 2022). Based on *in silico* studies, these proteins are hypothesised to be able to bind multiple VOCs, particularly stress-induced isoprenoids, with varying specificity (Giordano et al., 2021).

Thus, OBP-like proteins may facilitate the interaction of VOCs with intracellular receptor proteins or other signalling molecules (Russo et al., 2022). Indeed, with some analogy to the function of OBPs, proteins have been identified, whichbind to volatile phytohormones, i.e. the esters methyl salicylate (MeSA) and methyl jasmonate (MeJA) (Sheard et al., 2010; Gong et al., 2023). Receptors for non-volatile phytohormones are well known (e.g. Yan et al., 2018: nuclear-localised COI1 receptor of JA; Yan & Dong, 2015: NPR3 and NPR4 receptors for SA), as well as for the volatile phytohormone ethylene, i.e. ETR proteins localised to the endoplasmatic reticulum (Chen et al., 2002; Lacey & Binder, 2014). Furthermore, nucleus-localised TOPLESS-like proteins (TPLs) in tobacco were identified as transcriptional co-repressors that bind caryophyllene analogs and mediate VOC responses (Nagashima et al., 2019). The Karrikin Insensitive 2 (KAI2) receptor in *Petunia* was found to perceive the volatile terpenoid (−)-germacrene D, triggering a signalling cascade affecting plant fitness (Stirling et al., 2024)

If the substrate range of OBPs is broader than the specific compounds studied so far, structurally similar compounds may also get bound by them. Interestingly, many insect pheromones have various isoprenoid structures, including monoterpenes, sesquiterpenes, and diterpenes (Karlson, 1969; Moiseenkov et al., 1984). Notably, some insects produce

specific terpenoid pheromones, such as (*E*)-β-ocimene in *Heliconius melpomene* butterflies (Darragh et al., 2019) and *cis*-verbenol in *Ips confusus* bark beetles (Silverstein et al., 1967). In addition to terpenoids, many carboxylic acid esters also act as pheromones in insect species (Francke & Schulz, 2010; Wang et al., 2014; Naka & Fujii, 2020), highlighting the diversity of chemical structures used in insect communication with similarities to plant produced ones.

Future studies need to elucidate whether OPBs are involved in perception of insect pheromones by plants and how species-specific putative plant receptors of insect pheromones are.

5.2 Selective Nature of Pheromone Priming in Pine

Based on the results obtained from this thesis and a former study (Bittner et al., 2019), pheromone-mediated priming might have evolved as a selective defence mechanism of pine trees targeting directly *D. pini* oviposition and eggs. While it has been known prior to my thesis that exposure to sawfly sex pheromones primes pine trees and reduces the survival rate of *D. pini* eggs (Bittner et al., 2019), **Chapter 2** of this thesis presents evidence of counter-adaptations by *D. pini* females against pheromone-primed defences; the females prefer egg deposition on non-primed trees over primed, pheromone-exposed ones. Further investigation on the extent of pheromone-primed defences in pine trees (**Chapters 2** and **3**) showed that the effects of pheromone priming are restricted; the priming does not extend to defences against *D. pini* larvae (**Chapter 2**), nor does it influence indirect defences against the eggs. Exposure of pine to sawfly pheromones did not affect the emission of volatile organic compounds (VOCs), so that egg parasitoids of *D. pini* were not attracted by the odour of pheromone-exposed pine (**Chapter 3**).

The **Chapter 2** finding that exposure of pine to *D. pini* pheromones does not affect the larval performance indicates that the pine's response to the pheromone primarily acts as an oviposition deterrent upon contact at the behavioural level. If eggs are laid on pheromone-exposed pine needles in spite of the deterrent effect, the eggs suffer a higher mortality on these needles than on non-exposed ones (Bittner et al., 2019). The pine's response to the sawfly pheromone did not influence the development of the larval offspring. This decoupling of effects on the oviposition behaviour from larval viability suggests that the choice of the oviposition site is highly important for successful reproduction. While it was found in my thesis that ovipositing *D. pini* females exhibit a preference for non-primed trees, former studies showed that the subsequent development of their larval offspring is not directly linked with their oviposition preference (Pasquier-Barre et al., 2000; Barre et al., 2002). Likewise, we did not detect growth differences of *D. pini* larvae on pheromone-treated and untreated trees. However, performance of larvae was dependent on whether pine had received *D. pini* eggs or not prior to larval feeding. Larvae feeding upon previously egg-laden pine gained less weight than those on egg-free pine (Chapter 2). This latter result confirms a finding obtained in a previous study by Beyaert et al. (2012). Hence, pine responses to *D. pini* egg deposition intensify pine defence against *D. pini* larvae.

When addressing the question why the effect of pheromone exposure of pine is restricted to deterrence of ovipositing sawfly females and reduction of egg survival, the following needs to be

considered: The studies on pine – pine sawfly interactions showed that (i) egg-mediated priming exhibits a pronounced defence effect on larval development, while (ii) pheromone priming already intensifies pine defences against the ovipositing females and their eggs. Thus, a pheromone-mediated priming effect that targets also the larvae might be redundant and at the cost of plant performance. The resilience of specialist larvae, such as those of *D. pini*, to these priming effects is noteworthy. While pheromone priming initially makes the plant less preferred by female sawflies for oviposition, the performance of larvae on egg-free, pheromone-primed plants was not affected as shown in Chapter 2. Our findings imply that these larvae possess adaptive mechanisms enabling them to cope with or even circumvent the effects that exposure to pheromones elicits in pine. This adaptability could be attributed to their specialised feeding habits and evolutionary history with their host plants. It will be interesting for future studies to investigate a potential cross-talk between egg-elicited and pheromone-elicited pathways to assess any synergistic or antagonistic interactions between these priming effects.

The findings presented in **Chapter 3** showed no evidence of pheromone-mediated effects on the emission of pine volatiles relevant for egg parasitoid attraction. Plants are expected to balance the resources invested in direct and indirect defences (Agrawal & Fishbein, 2006; Singer et al., 2012; Agrawal, 2011). It is tempting to suggest that activation of direct defences may help the plant deterring herbivores upon contact, as indicated by the results of Chapter 2**.** Exposure of pine to pheromones will provide a pheromonal "cloud" close to pine needles, but does not provide information about the precise site of attack by the sawfly. Deterrence of ovipositing sawfly females by a pheromone-elicited change in contact cues of pine needles may be an advantageous direct defence strategy targeting the initial phase of infestation.

The Chapter 3 study suggests that activating indirect defences, such as the attraction of egg parasitoids in response to pheromone perception alone, might not be necessary for pine trees. This suggestion is based on the finding that *D. pini* sex pheromones *per se* are already attracting egg parasitoids (Hilker et al., 2000). If *D. pini* sex pheromones would induce pine trees to release an odour attractive to the parasitoids, that could interfere with the parasitoids' ability to detect their primary attractive cues, the pheromones. Our study on the effects of pheromone exposure on pine's indirect defences against sawfly eggs revealed that *C. ruforum*, the parasitoid targeting pine sawfly eggs, showed no preference for odours from pheromone-exposed trees compared to unexposed ones.

Our Chapter 3 study also revealed significant alterations in the emission rates of (*E*)-β-farnesene and (*Z*)-β-ocimene in response to egg deposition by *D. pini*. Already 24 hours after egg deposition, the emission rates of these two terpenes were enhanced when compared to those of egg-free pine. Higher emission rates of *(E*)-β-farnesene from egg-laden than from egg-free pine are crucial for attraction of the egg parasitoid *C. ruforum* to egg-laden pine (Hilker et al., 2002). However, it is interesting that previous studies found that the egg parasitoid is only attracted to pine odour 72 hours after egg deposition (Schroeder et al., 2008; Beyaert et al., 2010), but not earlier after egg deposition. These previous studies were conducted with pine twigs from mature trees, whereas the Chapter 3 study was done with very young pine trees. It will be interesting to figure out whether the egg parasitoid would also be attracted to odour of young pine trees 24 hours after egg deposition. The Chapter 3 study also showed that pheromone exposure of pine did not further enhance the emission profile of terpenoids from egg-deposited pine trees. This apparent lack of synergistic enhancement with combined, sequentially applied external cues (pheromones and eggs) suggests that the signalling pathways activated by pheromone exposure

and oviposition might either converge at a point where they reach a maximum capacity for terpene production or operate in a manner that prioritises one stimulus over the other.

Future research could focus on the precise temporal changes of terpene emissions in response to both pheromone exposure and oviposition. This would involve more frequent sampling and analysis within the first 24 hours post oviposition to better understand the timing and interplay of direct and indirect defence mechanisms.

5.3 Needle Age Influences Anti-Herbivore Defence in Pine

The developmental stage of a plant is a key factor in shaping its defence response to herbivory (Van Dam et al., 2001; Boege & Marquis, 2005; Hare, 2010). Studies show that both plant and leaf age play a crucial role in determining the effectiveness of these defences against pathogens and herbivores. Young leaves generally exhibit stronger defences compared to older leaves, with higher concentrations of defence-related compounds and increased resistance to pathogens (Dadáková et al., 2020; Picanço et al., 2023).

Chapter 4 of this thesis demonstrates that *D. pini* females prefer to lay eggs on one-year-old pine needles, where egg survival is higher compared to the current year's young needles. The results shown in this chapter provide for the first time information on the survival rates of eggs on two different needle age groups of pine. The oviposition preference of *D. pini* for older needles supports the preference-performance hypothesis, suggesting that oviposition choices are driven by conditions most favourable for offspring development (Mayhew, 1997, Gripenberg et al., 2010, Jones, 2022).

In our study, the oviposition choices by female sawflies corresponded to the distinct feeding preferences in larvae. Early instar larvae tended to favour young needles, whereas later instars preferred older needles, which led to greater weight gain. Conifer-feeding sawfly larvae appear to exhibit a preference for old needles over young ones, as noted for several species of the genus *Neodiprion* (Ikeda et al., 1977; Giertych et al., 2007) and for *D. pini* (Whitehill et al., 2023). Hence, the outcome obtained by the larval feeding preference assay in this thesis partly aligns with findings of previous studies. However, field conditions may influence oviposition and feeding preferences, as factors like enemy-free space for eggs and larvae could drive oviposition and feeding site selection (Björkman et al., 1997). The findings presented in Chapter 4 underscore the benefits of adapting oviposition and feeding behaviour to the host plant's foliage age, thus optimizing egg survival and larval performance, a critical insight highlighted in this thesis.

Additionally, young Scots pine needles showed stronger constitutive defences, including significantly higher levels of SA and ABA compared to old needles. This trend reflects the high defensive capability of juvenile tissues, which is crucial for the survival of young plants under high herbivory pressure (Oñate & Munné-Bosch, 2008). The study presented in Chapter 4 used young pine trees. Older Scots pine trees, which also possess both young and old needles, might display a similar pattern of hormonal and chemical defence allocation. Studies on angiosperms showed both plant age- and tissue age-dependent differences in hormonal responses to stress (Wasternack & Hause, 2013; Mao et al., 2017; Berens et al., 2019; Rankenberg et al., 2021). However, only limited knowledge seems to be available about how plant age and especially age

of long-lived plants affects the hormonal responses of mature leaves compared to those of freshly flushing leaves (Mao et al., 2017; Kanojia et al., 2021).

In our study, larval feeding induced JA, JA-Ile, and ABA in both young and old needles when compared to undamaged controls. SA levels were induced by larval feeding particularly in old needles when compared to controls. When comparing phytohormone levels of feeding-damaged young and old needles, these were similar for JA, but higher in young, feeding-damaged needles than old, feeding-damaged ones for SA and ABA. This suggests that while ABA and SA responses may vary with needle age, JA signalling might regulate defence responses independently of needle maturity. Future studies could further explore how needle age impacts the hormonal cross-talk in both juvenile and mature pines.

The higher trypsin inhibitor levels in young than one-year-old needles came along with reduced larval growth. This finding points to the importance of PIs in defending against herbivores (Gatehouse, 2011). While PIs are known to be inducible by plant damage (Van Dam et al., 2001), our study found that their activity in young needles did not change in response to herbivory, thus potentially providing a constitutively active defensive advantage. This finding is consistent with research indicating that juvenile pine needles generally exhibit stronger defences than mature needles, with higher concentrations of defensive secondary plant compounds (e.g. Erbilgin & Colgan, 2012).

In conclusion, Chapter 4 emphasises the importance of needle age in the defence mechanisms of Scots pine against herbivory. Future research could expand on this by investigating additional needle age groups to better understand how needle age affects chemical defences. It will be particularly interesting to figure out how constitutive and inducible low molecular secondary plant compounds and PI activities change in the course of needle aging. Moreover, molecular studies focusing on the pathways involved in the production of key phytohormones, such as SA, ABA, and jasmonates, will be crucial for elucidating their roles in mediating these responses. Examining gene expression related to these pathways in different needle ages can provide deeper insights into the adaptive strategies employed by *P. sylvestris* in response to herbivory.

5.4 Outlook

This thesis contributes to the understanding of how Scots pine (*P. sylvestris*) defends itself against herbivory by the sawfly *D. pini*, focusing on the role of pheromone-mediated priming and age-related needle defences. The findings not only highlight the complexity of plant-insect interactions but also open several research avenues.

One immediate direction for future research lies in dissecting the molecular mechanisms underpinning pheromone perception in plants. While this study has demonstrated that Scots pine can perceive and respond to sawfly pheromones by priming its defences, the exact molecular pathways involved remain largely unknown. Investigating how these pheromones are detected and identifying the downstream signalling pathways, would provide crucial insights into the evolution of plant sensitivity to insect semiochemicals. Specifically, exploring whether Scots pine and other conifers possess specialised receptors or signalling components for insect pheromones could enhance our understanding of cross-kingdom chemical communication.

Moreover, the specificity of the pheromone-primed response warrants further exploration. For example, it is unknown as yet whether also sex pheromones of closely related pine sawflies, which might occur in the forest at the same time as *D. pini*, also prime pine defences against sawfly eggs. This could be particularly relevant in ecosystems where multiple herbivores co-exist and compete for the same host plants. Understanding whether and how Scots pine distinguishes between the pheromones of different herbivore species would shed light on the selectivity of plant defence priming and offer broader ecological insights.

Another promising direction would be to assess the ecological consequences of these pheromone-mediated interactions in natural forest settings. While this thesis focused on controlled bioassays and lab-based experiments, which provide valuable insights into the mechanistic underpinnings of defence priming, translating these findings into a more ecologically realistic context is essential for understanding how these interactions play out in complex, multi-trophic environments.

Regarding the age-dependent defences of pine needles, further research could explore the trade-offs between growth and defence in different pine species, as well as across various ages of pine trees with varying needle longevity. Investigating the ecological and evolutionary pressures that shape needle longevity and defence investment across different conifer species could provide a more nuanced understanding of plant defence strategies.

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Curriculum Vitae

For reasons of data protection, the curriculum vitae is not published in the electronic version.