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Interactions between plants, herbivorous insect and predators: mechanisms and ecological importance

Ph.D. Thesis

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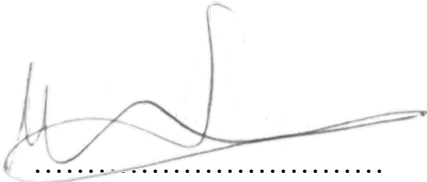
Annotation

The thesis describes the interactions between the three trophic levels from different perspectives. It focuses on the role of plants, herbivorous insect, and invertebrate predators and birds in trophic interactions. More specifically, it describes indirectly induced plant defence and communication with higher taxa and changes in predation pressure and insect communities in response to defensive chemical compounds released from herbivory-damaged plants.

Declaration

I hereby declare that I am the author of this dissertation and that I have used only those sources and literature detailed in the list of references.

České Budějovice, 7th of June 2022



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Anna Mrázová

This thesis originated from a partnership of Faculty of Science, University of South Bohemia, and Institute of Entomology, Biology Centre of the ASCR, supporting doctoral studies in the Zoology study programme.



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List of papers and author's contribution

The thesis is based on the following papers (listed chronologically according to the order in the thesis):

- I Mrazova, A.*, Sam, K., Amo, L. 2019. What do we know about Birds' use of plant volatile cues in tri-trophic interactions? Current opinion in insect science 32:131-136.
AM contributed to Investigation (75%), Writing - original draft preparation (75%), Writing - review & editing (33%), and funding acquisition (20%).
- II Amo, L.*, Mrazova, A., Saavedra, I., Sam, K. 2022. Exogenous application of methyl jasmonate increases emissions of volatile organic compounds in Pyrenean oak trees, *Quercus pyrenaica*. Biology 11:84.
AM contributed to methodology (30%), Investigation (25%), Data analyses (75%), Writing - original draft preparation (30%), Writing - review & editing (25%), and funding acquisition (40%).
- III Mrazova, A., Sam, K.* 2019. Exogenous application of methyl jasmonate to *Ficus hahliana* attracts predators of insects along an altitudinal gradient in Papua New Guinea. Journal of Tropical Ecology 35:157-164.
AM contributed to methodology (65%), Investigation (35%), Data analyses (50%), Writing - original draft preparation (75%), and Writing - review & editing (50%).
- IV Mrazova, A.*, Tahadlova, M., Rehova, V., Sam, K. Specificity of induced chemical defences of two oak species influence differently insect communities and predation. Manuscript.
AM contributed to methodology (85%), Investigation (25%), Data analyses (50%), Writing - original draft preparation (100%), and Writing - review & editing (30%).
- V Mrazova, A.*, Sam, K. Naïve Blue tits (*Cyanistes caeruleus*) and Great tits (*Parus major*) search for food preferably in herbivore damaged, than in undamaged shrubs. Manuscript.

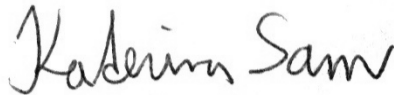
AM contributed to methodology (70%), Investigation (100%), Data analyses (85%), Writing - original draft preparation (75%), and Writing - review & editing (50%).

VI Sam, K.*, Jorge, L. R., Mrazova, A. Application of methyl jasmonate attracts predators to conspecific neighbouring Oaks, but not to trees of a sister species. Manuscript.
AM contributed to methodology (20%), Data analyses consultations (30%), and Writing - review & editing (20%).

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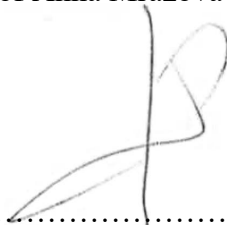
Katerina Sam, the supervisor of this thesis and first author and co-author of **Chapters I-VI**, fully acknowledges the contribution of Anna Mrazova as the first and co-author and her contributions as stated above.



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Mgr. Kateřina Sam, Ph.D.

Luisa Amo, the first author of **Chapter II** fully acknowledges the major contribution of Anna Mrazova in the presented manuscript.



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MSc. Luisa Amo, Ph.D.

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General introduction

Tri-trophic interactions

Trophic interactions, i.e., trophic food-chain, is a process during which the energy from the bodies of individuals of one species is transferred to those of different species. A trophic level then pertains to a position of a group of organisms in a food chain. To sustain stability of an ecosystem, signalling and communication pathways are needed between the trophic levels (Paine, 1966). Due to the complexity of the trophic chains, a vast body of research demonstrates that many ecological processes can only be understood from a tri-trophic viewpoint (at least), that is, one that moves beyond the pairwise interactions of neighbouring trophic levels. Tri-trophic interactions can generally represent any three trophic levels (e.g., plant-pollinator-predator of pollinating insects). However, interactions between plants, arthropod herbivores, and natural enemies dominate the terrestrial ecology of our planet (Paine, 1966, 1980). Therefore, for the purposes of this thesis, when discussing tri-trophic interactions, I will always refer to interactions between plants, herbivores, and predators.

In the tri-trophic systems, indirect interactions often occur then the effect of one species to another is mediated by a third species. In our study system, the mutualistic relationship that developed between plants and predators of herbivorous insect sheds new light on the issue of trophic communities. Predators (and also pathogens or parasitoids) that attack herbivorous arthropods can benefit plants by eliminating the feeding of herbivorous arthropods or making it more difficult (Baldwin & Schultz 1983; Rhoades 1983). It is thought that many plant traits, enabling indirect interactions, have evolved in response to this mutualism. Despite the we unambiguously know that natural enemies are attracted to plants fed on by their prey or hosts, it is hard to prove that the plants actively developed a

palette of mechanisms favouring the recruitment of natural enemies (Heil 2014; Dicke & Baldwin, 2010; González-Teuber & Heil, 2009). An alternative explanation is that some of the traits are inevitable result of feeding damage by the herbivore, or a by-product of a diverse defence against herbivory.

From plants to predators and back: bottom-up and top-down control

Plant-insect interactions are arguably the key processes maintaining biodiversity (Poelman et al., 2008) and ecosystem functioning (Metcalf et al., 2014) in terrestrial ecosystems. Although some plant-insect relationships, such as pollination, are mutually beneficial, the most common plant-insect interactions are antagonistic, involving insect herbivory and plant protection against the herbivorous damage (Gatehouse, 2002). Specifically, in the tri-trophic systems, while the nutrients contained in leaves and plant defences determine the abundance of herbivorous insects feeding on plants (from the bottom), predators and parasitoids, in turn, affect herbivorous insects negatively (from the top) (Mooney et al., 2010). Bottom-up and top-down forces interact passively and actively. Thus, trophic cascades are controlled by plants through communication across trophic levels and plants act as mediators of both, bottom-up and top-down forces acting upon herbivore insects, and other arthropods. A passive effect of bottom up-control is for example any effect of the plant that reduces the growth rate of the herbivore makes it more prone to predation (the slow-growth high-mortality hypothesis (Feeny 1976)). Actively, plants exert control upon arthropods by releasing chemical defence compounds which (1) directly repel insects via unpalatable

compounds or (2) indirectly attract predators of herbivorous insects (Mooney et al., 2010).

Plants interact with other levels of the trophic cascade through constitutively expressed mechanisms (Chen, 2008) which can be mechanical or chemical (e.g., leaf structure, colour, secondary metabolites) or induced upon attack (e.g., semiochemicals, herbivore-induced plant volatiles, trichomes; Mithöfer & Boland, 2012; Bandloy et al., 2015). It is important to note, that some defence mechanisms (e.g., trichomes) can be constitutive, as well as induced (Bloomer et al., 2014). Constitutive defence mechanisms are typically assumed to be costly, diverting resources away from growth and reproduction. Thus, investment into permanent protection (i.e., constitutively) is advantageous in a constantly challenging environment with abundant herbivorous insects or at places where plant biomass is “expensive” to produce. Inducible defences, on the other hand, are triggered only when needed and allow plants to avoid high-level defensive investments unless required. Therefore, plants relying on induced defences are in advantage in environments where the presence of herbivorous insects is unpredictable to some extent (Shelton, 2004; Agrawal, 1999).

Chemical inducible plant defences act directly by synthesis of chemical compounds making the leaf tissue unpalatable or poisonous (e.g., Chen, 2008; Mithöfer & Boland, 2012; Dicke et al., 2009) or indirectly by emitting volatile organic compounds trackable by various predatory taxa (e.g., Dicke et al., 2003; Heil, 2014; Mrazova & Sam, 2018, 2019 – **Chapter III**; Mrazova et al., 2019 – **Chapter I**) (Fig. 1). As discussed above, the emission of the organic compounds may be active or passive. Nevertheless, the mechanism, the effectiveness of the indirect induced

General introduction

defences relies not only on the inducibility of the plant itself but also on the diversity and abundance of predators in the given habitat and their ability and willingness to respond to the plant cues.

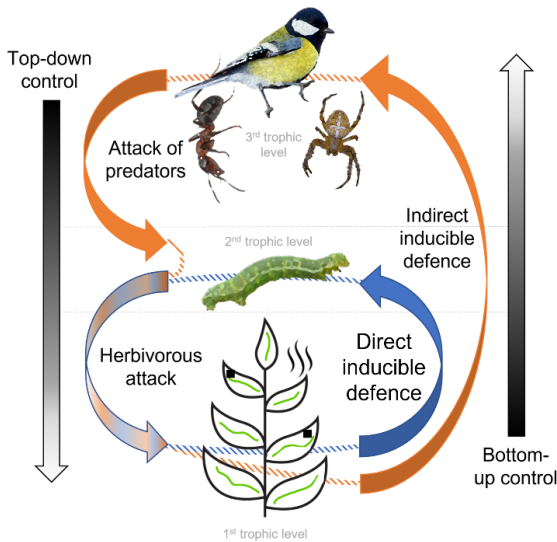


Figure 1: Direct and indirect chemical inducible plant defence: To control herbivore pressure from the bottom-up, the plants attacked by arthropods release non-edible chemicals that directly discourage herbivorous insects from feeding. Alternatively, in indirect defence, plants emit herbivore-induced plant volatiles (HIPVs) acting as a cue that leads predators of herbivorous insect to herbivore-rich plants (A. Mrazova, 2022).

Indirect inducible defence

Crying for help hypothesis depicting indirect inducible defence in plants, from plant-arthropod predator communities' point of view, was described by Baldwin and Schultz and Rhoades in 1983. This study described that herbivore-induced plant volatiles (HIPVs) released by plant under herbivorous attack can act as mediators between herbivore-damaged plants and natural enemies of herbivorous insect. Thereafter, from the late 80s, studies describing more sophisticated systems including various plant and animal taxa based on the hypothesis started to appear. Two groups of natural enemies with different strategies that increase fitness of plants under herbivorous attack were described. First, predators killing the prey immediately, thereby ridding the plant of herbivorous insects instantly (Dicke & van Loon, 2000). Second, parasitoids, which lay eggs into

herbivorous which then often continues feeding on the plant. Studies of solitary Diptera and Hymenoptera have, however, shown that the parasitized host consumes less biomass than the unparasitized host (Rahman, 1970; Harvey et al., 1999; Dicke & van Loon, 2000).

Among others, the Crying for help hypothesis has been described as valid for predatory mites (Dicke et al., 1988), parasitoid wasps (Turlings et al., 1990), predatory bugs (Drukker et al., 1995), predatory lady beetles (Ninkovic et al., 2001), and nematodes (Rasmann et al., 2005). In 2008, Mäntylä et al. proved that also vertebrate insectivores, represented by birds, can be guided by chemical signals emitted by herbivory-damaged plants (for detailed information on the topic, see review by Heil, 2014).

Although the research of the attraction of insectivorous birds to HIPVs has emerged in 14 years ago, their role in bird-herbivore-plant interactions is understudied. Specifically, the issue of birds' ability to use volatile cues to locate insect prey has been studied only superficially. Moreover, so far published studies have presented rather contradictory results (**Chapter I**).

Plant-plant volatile communication

Plants are able to communicate with their surrounding via electrical signalling, shared mycorrhizal networks and volatile organic compounds. This diversity of communication pathways allows plants to communicate not only with a range of other organisms (for example soil microbes (Wenke et al., 2010), fungi (Bonfante & Genre, 2015), insects (**Chapters III, IV**, De Moraes et al., 1998), and vertebrates (**Chapters I-VI**, Leonard & Francis, 2017) but also with other plants (**Chapter VI**).

It could be beneficial for neighbours of a plant under herbivorous attack to receive a signal that warns them against increased abundances of herbivores nearby, so that the undamaged neighbouring plant could launch a defensive reaction before the attack itself. The same species recognition hypothesis predicts that plants respond more effectively to signals emitted by close relatives (Karban et al., 2013). According to the mutual benefit hypothesis, communicating herbivory risk is beneficial to individual emitters regardless of their relatedness to recipients this exchange of information promotes the movement of herbivory away from the emitter or patch of emitter neighbours (Bruin & Dicke, 2001; Heil, 2014; Heil & Karban, 2010).

Plant-plant volatile communication showed to be very complex due to specificity of signalling compounds to a particular plant species or genotype, or to the herbivore species that induces their release by feeding on the plant (Douma et al., 2019). Most studies of plant-plant communication to date have focused on species-specific responses (e.g., Dolch & Tschardtke, 2000; Kalske et al., 2019; Karban et al., 2013; Karban et al., 2014). However, interspecific plant communication has also been documented (e.g., Farmer & Ryan, 1990; Glinwood et al., 2004; Karban et al., 2000; Oudejans & Bruin, 1994) in several plant genera (Peñuelas & Llusà, 2004).

As evidenced above, the number of studies examining plant-plant volatile communication accumulated over the years. However, existing results have not yet found convincing pattern (Baldwin & Schultz, 1983; Dicke & Bruin, 2001; Rhoades, 1983) to adopt description of various aspects of plant communication (Dicke et al., 2003; Pickett et al., 2003).

Methyl jasmonate

Regulation of many physiological processes in plants, including communication and mediation of plant responses to both, abiotic and biotic stresses are regulated by an octadecanoid-based signalling pathway (Delker et al., 2006) involving jasmonic acid and its precursors and derivatives, referred to as jasmonates including methyl jasmonate (Wasternack, 2007). Jasmonates are a class of oxidized lipids (oxylipins) derived from α -linolenic acids (Fonseca et al., 2009) and act on gene expression to slow plant growth and redirect metabolism to produce defence molecules and repair damage (Campos et al., 2014).

In response to plant tissue damage, jasmonic hormones are produced when the plant is attacked by pathogens, oviposition, herbivores, or mechanical stress. In response to a local stimulus (i.e., herbivorous damage), damaged tissues were also observed to produce temporary defence molecules (Green & Ryan, 1972). An external application of jasmonates (jasmonic acid or its volatile derivative methyl jasmonate - MeJA) in low concentrations to plant leaves has been shown to induce defensive responses in plants (**Chapters I-IV, VI**). The MeJA application causes temporal changes in plant defensive acting by producing increased (i) amounts and newly synthesized defensive compounds (Green & Ryan, 1972); (ii) densities of mechanical plant structures (e.g., trichomes and spines; Boughton et al., 2005).

The effect of external application of MeJA on insect communities on treated host plant remains conflicting despite the increasing number of studies. So far, published studies refer to (1) no effect of external MeJA application to performance, mortality, or insect abundance (e.g., Williams et al., 2017; **Chapter IV**), or (2) significant repelling effect (e.g., Tan et al.,

2011). If MeJA has no effect on total abundances of insects on a host plant, a change in the ratio of individual insect guilds might be the reason. While herbivorous insect can be repelled by effect of defence mechanisms triggered in plants by MeJA application, invertebrate predators might be, in contrast, according to Crying for help hypothesis, attracted by induced chemical volatile compounds. Thus, the resulting insect abundances may remain unchanged.

Birds were attracted to MeJA-treated plants in two out of four so far published studies. Thus, the induction of plants with MeJA in field manipulative experiments examining birds' ability to use volatile cues to find their prey was considered unreliable. It has been suggested that such inconsistency might be caused by differences in defensive volatile spectra emitted by naturally (with real caterpillars) and MeJA-induced plants (Baldwin, 1988; Moreira et al., 2012; Papazian et al., 2019). Above mentioned studies, however, differed in study site habitats, plant, larvae, and bird species studied, and doses of MeJA solution used.

According to a comparative study revealing the use and usefulness of artificial herbivory induction in plants, of the 46 studies found, 33 (72%) reported a significant difference between the response to artificial and natural herbivore damage in at least one of the included statistical tests (Lehtil & Boalt, 2008). However, **Chapter II** shows that the chemical profile of Pyrenean oak (*Quercus Pyrenaica*) released by caterpillar-infested and MeJA-treated trees does not significantly differ (**Chapter II**).

Olfaction in birds

Birds use their excellent vision to navigate, hunt and orient themselves in space. Bird' vision was considered the dominant sense, therefore, only little emphasis has been paid on the study of their olfactory abilities, even though research on the sense of smell in birds is more than 60 years old. Although it was known that birds with large olfactory bulbs (e.g., petrels, vultures) use olfaction in feeding behaviour, until the first half of the 1960s, most studies considered birds with small olfactory bulbs (e.g., songbirds) anosmic (Roper 1999).

The avian nasal cavity consists of three chambers. In the uppermost chamber, the olfactory epithelium with olfactory receptors is developed in all birds and is composed of differentiated nerve cells. Signals detected by the epithelium are conducted by the olfactory nerve and processed in the cerebellum (Hartwig 1993). The olfactory mucosa of several bird genera is perfectly developed (Veselovsky 2001). Petrels (Procellariiformes) locate food-rich sites by detecting the odour of carboxylic acids contained in the crustacean bodies (Grubb 1979). Blind condor chicks kept in zoo can perfectly recognize their caretaker under the smell of ethyl mercaptan (Hediger 1968). However, recent studies have evidenced that even birds with small olfactory bulb-body size ratio can detect odours in different contexts.

Female common eiders (*Somateria mollissima*) rub their eggs with a smelly excrement to prevent predation on the nest from crows and gulls (McDougall & Milne 1978). During the mating season, bills of the same species emit a smell of tangerines to attract partners. Male mallards (*Anas platyrhynchos*) are attracted by the scent of females given off by the costal gland during the breeding period (Jacob et al., 1979). Pigeons, for example,

use their sense of smell to navigate in space, creating scent maps and locating nesting sites with perfect accuracy (Guilford et al. 1998). Bridled terns (*Pygoscelis antarcticus*) can detect dimethyl sulphide molecules in the air released from high-productivity sites in the oceans (Amo et al. 2013).

Over the last 14 years, we recorded increasing number of studies examining the role of insectivorous birds in tri-trophic interactions. Although the results of research on bird's use of herbivore-induced plant volatiles are still conflicting in some of the aspects, they proved Crying for help hypothesis valid also for insectivorous birds.

Aims and the scope of the thesis

This thesis focuses on mechanisms and ecological importance of interactions in tri-trophic systems. In 2019, with cooperation of two of my colleagues, I wrote a review summarizing the current knowledge on this topic (**Chapter I**). Later I conducted several complementary experiments in nature, and in aviaries, to explain the mechanisms in tri-trophic systems. I executed field manipulative experiments in which I induced chemical defences of plants using (1) plant hormone Methyl jasmonate (**Chapter IV**) or (2) lepidopteran larvae in the Czech Republic (**Chapter V**), and Papua New Guinea (**Chapter III**) and studied ability of insect predators to detect chemical or visual cues provided by induced plants. My results consistently show the importance of communication between distinct trophic levels – plants and predators of herbivorous insect. I further demonstrated that external application of MeJA induces a chemical response comparable to the natural induction of real herbivory (**Chapter II**) in Pyrenean oaks. Finally, I focused on air-borne communication

between plants via induced volatile compounds. I found that the application of MeJA triggering a defensive reaction in oaks attracts predators to conspecific, but not to heterospecific neighbouring individuals (**Chapter VI**). Overall results of my research proved, among others, that: (1) Even birds with small olfactory bulbs (Great and Blue tits specifically) can smell and be guided by odours in various contexts of life; (2) Birds can smell plants damaged with herbivorous insects; (3) Birds can also distinguish between chemical and visual signals provided by herbivory-damaged plants; (4) The use of methyl jasmonate as an inducer of plant chemical response seems to be an appropriate tool in manipulative experiments; (5) Conspecific tree species with advanced chemical defence communicate via volatile compounds.

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Chapter I

What do we know about birds' use of plant volatile cues in tri-trophic interactions?

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What do we know about birds' use of plant volatile cues in tri-trophic interactions?

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Abstract

The first study showing that birds can smell herbivore-induced plant volatiles was published ten years ago. Since then, only 12 studies have been published, showing contradictory results. This review evaluates the role of birds in relation to the crying for help hypothesis and their use of olfactory cues. In accordance with the methodologies used in previous studies, we herein provide a summary of experimental approaches and describe the advantages and disadvantages of experiments conducted in nature versus aviaries. Moreover, we recommend experimental methodologies which lead to a deeper knowledge of the topic, including reflection on the induction of plant defenses and adaptations of birds. Finally, we propose some interesting questions for future research to direct further studies towards a thorough and accurate description of birds' roles in tri-trophic interactions.

Introduction

A 350-million-year-old evolutionary race between herbivorous insects and plants resulted in the ability of plants to communicate with predators of insects via chemical signals: the indirect induced defense [1]. The release of herbivore-induced plant volatiles (HIPVs) is often mediated by the plant hormone methyl jasmonate, synthesized by a plant in response to stress caused by the combination of wounds and elicitors contained in the saliva of herbivores [2].

According to the ‘crying for help’ hypothesis [3,4], predators and parasitoids can use HIPVs as a cue to find their herbivorous prey or hosts on the host plant, thus helping the plant to cope with herbivores. Although attraction to herbivore-induced plant volatiles has been well-documented for arthropod predators and parasitoids (predatory mites, parasitoid wasps, predatory bugs, nematodes, etc.) [5], research on other critical predators, insectivorous birds, has emerged only in the last 10 years [6].

Birds are important predators of herbivorous insects [7,8] and have been shown to effectively reduce insect abundance in most studied habitats. While their role in tri-trophic interactions is, therefore, very important, research on bird-herbivore-plant interactions is still in its infancy. Specifically, the issue of birds’ ability to use volatile cues to locate insect prey has received limited study. Moreover, 19 experiments published in 14 studies (two unpublished) have presented rather contradictory results (Table 1). This review provides an overview on the role of birds in HIPV-mediated tri-trophic interactions, summarizes existing knowledge, and compares methodologies that have been used. Moreover, we present a likely direction of research for tri-trophic interactions, including birds as predators, and suggest experiments that are required for a deeper understanding.

Olfaction in birds

Although our understanding of the olfactory system in predatory insects is still incomplete, a series of simple experiments was able to provide much critical information. Olfactometers, wind chambers, Y-shaped tunnels and other simple devices enabled the researchers to experimentally isolate olfactory cues and the insects' responses to them in preference assays. The mechanisms of hunting in invertebrate predators naturally led to the high number of studies and deep knowledge of their olfactory abilities.

Birds use their excellent vision for navigation, hunting and orientation in a space. The study of their olfactory capacities, however, has not been emphasized, even though research into the sense of smell in birds is more than 60 years old. The very basic question at the beginning was: "Can birds smell?" For the first half of the 1960s, most studies asserted that they cannot. However, in 1967, Wenzel [9], focusing on the variance in anatomy of olfactory bulbs in different bird species, showed that this was not the case. By exposing birds to several chemical stimuli, he showed that "the olfactory structures in birds do have some functional status". During the following 50 years of research, it was shown that some birds can be guided by volatile organic compounds spread in the atmosphere on their way home (e.g. pigeons; [10]) and others use olfaction for finding places rich in food (e.g. Procellariiformes; [11]). Even birds with very small olfactory bulbs (e.g. Passerines) can detect odors in different, mostly social, contexts [12–14]. The ability of birds to use their sense of smell in common life situations led scientists ask whether birds could respond to HIPVs to find prey [3–6].

Birds as a part of the crying for help hypothesis?

Considering the fact that birds can use smell for navigation in different situations, the question is whether they can be guided by chemicals released from plants attacked by herbivores. The first evidence revealing that birds are attracted to insect-infested trees, without the need to see the insect or damage to the leaves, was provided by Mäntylä et al. [15]. They also explored whether birds use visual [16] or chemical cues [15] to discriminate between infested and uninfested trees. However, the mechanism underlying such discrimination was not elucidated until later, when Amo et al. [17●●] isolated chemical and visual cues of infested and uninfested trees in an aviary experiment to investigate the cues used by insectivorous birds. Their study showed that great tits (*Parus major*) preferred searching for food on an apple tree (*Malus silvestris* Miller; variety De Costa) emitting a strong chemical signal compared to a tree only providing a visual signal (i.e. trees previously infested by insects but from which both the insects and damaged leaves were removed). Thus far, an experiment by Amo et al. [17●●] is the only study where HIPV olfactory foraging cues have been offered to insectivorous birds both with and without visual cues. The attraction of insectivorous birds to infested trees may not be innate but it seems it can be learned. Hand-raised naïve great tits were not attracted to infested apple trees in an aviary experiment [18●●], although they were attracted to them after gaining experience (through training in aviaries) foraging for caterpillars in the same trees [17●●]. These results are consistent with the hypothesis that naïve, inexperienced, generalist vertebrate predators have no preference for HIPVs until they perceive odours in association with a successful foraging experience. With the exception of six experiments in aviaries, most experiments dealing with the ability of birds to discriminate between trees infested and uninfested with herbivorous insects have been performed in nature, where wild birds were allowed to attack the artificial prey on manipulated trees (Table 1). Despite the slowly increasing number of studies, results are still conflicting and fail to consistently describe a pattern.

Table 1

A list of studies where the response of birds to plants with actual (caused by caterpillars - Cater.), induced (Ind.), and simulated by mechanical damage of leaves (Mech.) was studied in nature or in aviaries. In some studies, an analysis of volatile compounds induced by the (simulated) leaf damage was included, and others indicated whether birds had experience with association of food and smell or were naïve to smell

Author	Year	Experiments		Herbivorous damage			Reaction		Different VOC		Bird species	Experience	
		Cage	Nature	Cater.	Ind.	Mech.	Neg.	Pos.	Yes	No		Exp.	Nave
Mäntylä	2004	1	0	1	0	0	0	1	NA		Willow warbler	1	0
Mäntylä	2008a	0	1	1	0	0	0	1	1	0	Blue and Great tit	1	0
Mäntylä	2008b	0	1	1	0	0	0	1 ^a	NA		NA	NA	
Mäntylä	2011	0	1	1	0	0	1	0	1	0	Pied flycatcher	1	0
Mäntylä	2014	0	1	0	1M	0	1	0	1	0	NA	NA	
Mäntylä	2014	0	1	1	0	0	0	1ns	1	0	NA	NA	
Mäntylä	2017	1	0	1	0	0	0	1	1	0	Blue and Great tit	1	0
Koski	2016	1	0	0	1O	0	1	0	1	0	Pied flycatcher	1	0
Koski	2016	0	1	0	1O	0	1	0	1	0	NA	NA	
Koski	2016	1	0	1	0	0	1	0	1	0	Blue and Great tit	1	0
Amo	2013	1	0	1	0	0	0	1	1	0	Great tit	0	1 ^b
Amo	2016	1	0	1	0	0	1	0	NA		Great tit	0	1
Mrazova	2017	0	1	0	1	0	0	1	1	0	NA	NA	
Mrazova	unpubl.	1	0	1	0	0	0	1	1	0	Blue and Great tit	0	1
Saavedra	2018	0	1	0	1	0	1	0	NA		Blue tit	1	0
Saavedra	2018	0	1	0	1	0	1	0	NA		Blue tit	1	0
Saavedra	2018	0	1	0	1	0	1	0	NA		Blue tit	0	1
Mrazova	in prep	0	1	0	1	0	0	1	1	0	NA	NA	
Sam	2014	0	1	0	0	1	0	1	NA		NA	NA	

^a Positive reaction only on trees from large forest fragments. ns, non-significant results; NA, without measuring; M, induced by MeJA; O, other compounds simulating herbivorous damage.

^b Hand-raised but trained to locate food on infested trees. The last two studies under the horizontal dotted line were conducted in the tropics.

Experimental approaches

The choice of experimental approach is essential to correctly test hypotheses. Currently, there are only a few ways of studying the extent of the participation of birds in tri-trophic interactions and the crying for help hypothesis. Unfortunately, none of the options described below will allow us to answer all questions related to the highly complex tri-trophic system. However, the combination of specific single experiments can provide us with a more realistic picture of the role of birds in plant – herbivorous insect – bird interactions in the future. Extra caution needs to be taken when considering the specific type of experiment for future studies.

Experiments in nature

Field experiments are the best choice to study patterns in nature, since they provide a more realistic picture. Unfortunately, field experiments do not easily allow control over variables such as the identity of birds, or for the separation of chemical and visual cues. The advantage of field observation is thus partly lost by necessary manipulation. Naturally, plants are eaten by a variety of herbivorous invertebrates in combination. Yet it is not confirmed whether birds react specifically to individual volatile blends (induced by a specific herbivore), or whether their attraction to a plant is increased by a combination of blends. The birds would naturally benefit from being able to react to mixture of blends, rather than specifically to each of them. However, a specific herbivore on a single plant species was used more often in natural experiments, and the herbivore's abundance was controlled by adding them to a host plant [6,7,16,17●●].

The reaction of birds to a mixture of real herbivores feeding on a plant has not been studied, although one study used a mixture of volatile blends, which were induced chemically or mechanically in natural conditions [19, see below]. To compare the difference between predator reactions toward plants providing none or some chemical signals (induced either chemically, mechanically or placing high

abundances of herbivore on plant), the predation rate is usually measured by a bait in natural conditions. Live bait offers more realistic data, but their disappearance only implies predation without revealing the identity of the predator. Comparatively, artificial caterpillars, representing a less natural bait, provide a direct quantitative measure of the bird-specific predation rate under field conditions [20]. In the field, researchers only have a limited ability to control the experimental conditions (e.g. it is difficult in practice to separate real herbivore induced visual and chemical cues under field conditions), which might significantly influence results.

Experiments in aviaries

Experiments performed in captivity allow for better control of ambient conditions and individual variation in reactions of conspecific or heterospecific bird species. Furthermore, more complicated experimental designs can be used in order to isolate visual and chemical cues [17●●]; for example, a spectrophotometer can be used to evaluate how visual changes (e.g. UV reflectance) affect how birds discriminate between infested and uninfested trees [16]. Aviary experiments are, however, more labor demanding, requiring a certified breeding area, intense daily care for the birds, and a long time for habituation of wild birds in cages or the training of hand-raised birds. It is also unknown how the naïve, hand-raised birds, are affected by such unnatural manipulation. In aviary experiments with captive birds, a bird is typically released in an aviary with two trees, one infested (or previously infested) and one uninfested by herbivorous insects, in order to analyze the attraction of birds to both trees. This is achieved by measuring the first choice (i.e. first visited tree), the proportion of visits to the trees, and the proportion of time spent searching on trees. Specific experimental conditions, the size and location of aviary, and the physical condition of birds may play a role that requires consideration.

Induction of volatile organic compounds (VOCs) for plant defence

Recent studies have also used the induction of plant volatile organic compounds (VOCs) by a plant hormone, methyl jasmonate (MeJA) [21●,22●,23]. The application of MeJA on plant leaves activates various chemical reactions that lead to secretion of VOCs [24]. Two out of the three studies showed a positive reaction of birds to MeJA-treated trees [21●,22●] and thus concluded that birds are attracted to chemically active plants. One study did not support the preference of birds for MeJA-treated plants [23]. In all three studies, the mixture of compounds released by MeJA-treated plants differed from the VOCs released by plants damaged by herbivorous insects [21●,22●,23,25].

Only one study tested the response of birds to a completely artificial blend of VOCs [26], which partly mimicked the blend of volatiles released by defoliated mountain birches (*Betula pubescens ssp. czerepanovii*). Some of the compounds used in this study [26] were positively associated with higher predation rates ((E)-DMNT and linalool). However, natural VOCs and HIPV blends are much more complex than the VOC solutions used in the experiments. Furthermore, the ratios of the compounds were not precisely the same as those emitted by herbivore-damaged mountain birch, which may explain why the birds did not react to them. The use of an artificial blend of VOCs and HIPVs thus remains problematic. To resolve the disparity between experiments testing bird responses to real herbivores on a plant versus experiments using induced volatile compounds, it might be very important to use the precise volatile profile (including ratios) in experiments. If wild birds already learned to associate prey with a particular odor, even slight differences between the natural and experimental odors might prevent the experimental odor from being recognized as the learned cue. Further studies should investigate natural blends of experimental plant volatiles and carefully consider both how it differs from an artificial blend and the consequences of its use.

Behavioral plasticity of birds

The specificity of HIPV emission in relation to both plant and herbivore species provides useful information to predators or parasitoids that are specialized on a species of prey or hosts. In these cases, the innate detection of HIPVs may be under strong selection, as naïve emerging parasitoids or predators may not begin to search for their specific hosts or prey at random (e.g. in Refs. [27,28]). In contrast, for generalist predators such as insectivorous birds, prey availability may change during the year due to differences in the phenology of the species and, therefore, insectivorous birds may show adaptive plasticity in their foraging behavior in response to changes in the distribution and abundance of their prey species (e.g. in Ref. [29]). Under these circumstances, birds are likely to learn to associate different odors with a food resource to maximize the success of foraging efforts. Optimal foraging theory expects that birds will plastically respond to changes in prey availability and prey more on larger and/ or more abundant prey. If we assume that the birds use olfactory cues, at least partially, to search for prey, then we should expect birds to learn to associate changes in HIPVs with changes in abundance of prey. The number of different HIPVs which were positively associated with a bird's interest in particular trees in existing studies (Table 2) supports the hypothesis that birds are able to re-learn their associations of different substances to rewards, and thus have a plastic response to quickly changing surroundings. The adaptive value of learning is expected to vary among bird species depending on their breadth of diet at both the herbivore and plant levels [30].

In many insectivorous bird species, parents care for their young even after leaving the nest, and thus fledglings have time to learn to associate arthropod presence with HIPVs (through social transfer [31]). However, only half of the studies are consistent with the hypothesis that the ability to associate HIPVs with food in birds is not innate [16,18●●]. A focus is, therefore, needed on experiments

investigating whether the ability to associate an odor with a particular food source is innate or learned in, at least some or all, birds. Further experiments are needed to deepen knowledge on how experience helps insectivorous birds find herbivore-infested trees, and whether the experience needed depends on the plant or the insect species that the birds are dealing with.

Methodological recommendations

There is no doubt that our understanding of multitrophic interactions is far from comprehensive. However, the study of such complex system(s) brings several difficulties. One of many challenges in elucidating the role of HIPVs in plant-herbivore-bird interactions is the issue of bird experience. Before establishing a new study, the advantages and disadvantages of the use of naïve or experienced birds should be considered. Hand-raised naïve birds represent an easily obtainable uninfluenced subject. However, if we consider that the ability of birds to use different HIPVs in locating prey is not innate, there is no other choice than to use experienced wild-captured birds in cage experiments. Work with wild birds in large aviaries, where two experimental trees can be physically accommodated, is not easy, as birds often ignore the trees and try instead to escape. Intense habituation to experimental conditions may decrease the problem. A potential issue with this approach is that such trials could influence the behavior of birds in many ways and thus bias the results such that they do not represent interactions in nature.

More studies are needed to investigate the possibilities of using artificial mixtures of volatile compounds or methyl jasmonate (MeJA) (to induce the volatile compounds similar to those induced by arthropods) in tri-trophic experiments with birds. Special care needs to be taken of outside factors influencing the production of volatile compounds, the similarity of the real and artificially produced blends, and in particular, the response of birds to these compounds, which should be considered with caution.

Interesting questions for future research

The study of multitrophic interactions involving predatory birds, herbivorous arthropods, and plants is still in its infancy. The final score of existing experiments speaking for and against the ability of birds to use olfaction when searching for insectivorous prey is 8:8 in temperate regions (16 experiments in 12 studies; Table 1). A further two experiments conducted in the tropics also supports active bird participation in the crying for help hypothesis. Future studies should involve additional bird species or additional herbivores with their host plants. Such experiments would allow us to understand whether the attraction of birds to infested trees is common among insectivorous birds and how it may depend on the degree of specialization of the bird species in feeding on a particular prey species hosted on one or more plant species. Such knowledge will be important for generalization, as the majority of the studies have been conducted on a limited number of bird species (four species, see Table 1). Further research may also answer ecologically relevant questions such as whether birds are able to discriminate between trees infested with low or high numbers of herbivores. Such information is needed to understand the functioning of the birds as insect predators and would be applicable in effective pest control.

Most likely, insectivorous birds take a range of prey species, which can vary in size, so there is not only some degree of specificity in learning where to forage, but also some degree of opportunism. All of this is likely to be based on the use of cues in multiple sensory modalities and it remains to be answered to which extent the sensory modalities play a role in search for prey. According to optimal foraging theory and the growth of nestlings [32] it would be beneficial for insectivorous birds if they could somehow discriminate among trees infested with different developmental stages of herbivores, such that they can locate the prey with the most profitable size. It has been shown that resident birds, which had longer experience

with local prey, were feeding larger insects to their offspring than were migrant birds [31]. It is currently unknown whether the different developmental stages of insect prey (which produce different odors [33]) can be also detected by birds only visually or with additional olfactory cues.

How far away can birds detect the HIPVs, how do abiotic factors influence the detection of HIPVs by birds, and can birds discriminate between the HIPVs of infested plants or those of neighbouring uninfested plants, are questions that need to be answered in the future to have a better understanding of the relevance of HIPVs for insectivorous birds. The fast recognition of novel HIPVs that has been demonstrated in naïve great tits suggests that birds can be excellent candidates for use in the biological control of insect pests, especially because of the high predation rates of birds compared to those of predatory arthropods [8]. Responses to these unanswered questions are likely to make important contributions, not only to our understanding of tri-trophic interactions at the individual level, but also to our understanding of multi-trophic interactions and population and community processes [8].

Table 2

A list of the main volatile compounds that statistically differ between infested and uninfested trees in studies of the attraction of birds to herbivore-damaged trees (MB, Mountain birch; SB, Silver birch; EWB, European white birch)

Author	Year	Plant species	Herbivore	HIPVs
Mäntylä	2008a	<i>Betula pubescens</i>	<i>Epirrita autumnata</i>	β -Ocimene, linalool, (<i>E</i>)-DMNT [(<i>E</i>)-4,8-dimethyl-1,3,7-nonatriene], β -bourbonene, <i>cis</i> -3-hexenyl acetate, nonanal, limonene, humulene, caryophyllene oxide, (<i>E</i>)- β -caryophyllene, <i>cis</i> -3-hexen-1-ol+(<i>E</i>)-2-hexenal, <i>cis</i> -3-hexenyl butyrate
Mäntylä	2014	<i>Betula pubescens</i>	<i>Epirrita autumnata</i>	Myrcene, limonene
Mäntylä	2017	<i>Pinus sylvestris</i>	<i>Neodiprion sertifer</i>	Isobornyl acetate, bicycloelemene, α -copaene, isogermacrene D, aromadendrene, α -humulene, α -amorphene, α -selinene, α -muurolene, α -pinene, camphene, β -phellandrene, β -elemene, (<i>E</i>)- β -caryophyllene, (<i>E</i>)- β -farnesene, germacrene, bicyclogermacrene, α -farnesene, γ -cadinene, δ -cadinene, germacrene-D-4-ol
Koski	2016	Artificial tree	<i>Epirrita autumnata</i>	Artificial blend of Hexane, (<i>E</i>)-DMNT [(<i>E</i>)-4,8-dimethyl-1,3,7-nonatriene], (<i>Z</i>)-DMNT [(<i>Z</i>)-4,8-dimethyl-1,3,7-nonatriene], linalool, α -ocimene, limonene,
Koski	2016	MB, SB, EWB	<i>Epirrita autumnata</i>	Artificial blend of hexane, (<i>E</i>)-DMNT [(<i>E</i>)-4,8-dimethyl-1,3,7-nonatriene], (<i>Z</i>)-DMNT [(<i>Z</i>)-4,8-dimethyl-1,3,7-nonatriene], linalool, α -ocimene, limonene
Koski	2016	<i>Betula pendula</i>	<i>Epirrita autumnata</i>	α -Pinene, sabinene, β -pinene, β -myrcene, 1,5,8-p-menthatriene, d-limonene, 1-hexanol, 1,8-cineole, terpinolene, linalool, α -terpineol, (<i>Z</i>)-DMNT, (<i>E</i>)-DMNT, α -cubebene, α -copaene, (<i>E</i>)-caryophyllene, β -cubebene, germacrene D, α -humulene, α -amorphene, α -farnesene, α -muurolene, γ -cadinene, δ -cadinene, (<i>E</i>)-2-hexenal, (<i>Z</i>)-3-hexenol, (<i>Z</i>)-3-hexenyl acetate, (<i>Z</i>)-3-hexenyl butyrate, (<i>Z</i>)-3-hexenyl 2-methylbutanoate, 1H-indole, (<i>Z</i>)-3-hexenyl isovalerate, (<i>E</i>)-2-hexenyl tiglate, benzeneethanol, benzeneacetonitrile
Amo	2013	<i>Malus pumila</i>	<i>Operophtera brumata</i>	α -Farnesene, dodecanal, 1,2,4 trimethyl benzene, 1-octen-3-ol, methoxy phenyl oxime, 1-nonene, 3-octanol
Mrazova	2017	<i>Salix cinerea</i>	NA - MeJA	α -Pinene, β -pinene, 3-carene, limonene, β -ocimene
Mrazova	unpubl.	<i>Ligustrum vulgare</i>	<i>Samia cynthia</i>	D-limonene, limonene-oxide, benzyl acetate

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Chapter II

Exogenous application of methyl jasmonate increases emissions of volatile organic compounds in Pyrenean oak trees, *Quercus pyrenaica*

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Exogenous application of methyl jasmonate increases emissions of volatile organic compounds in Pyrenean oak trees, *Quercus pyrenaica*

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Simple summary

The tri-trophic interactions between plants, insects, and insect predators and parasitoids are a dominant component of many terrestrial ecosystems. Within these interactions, many predators of herbivorous arthropods use chemical signals provided by the host plants when searching for prey. The exogenous application of methyl jasmonate (MeJA) often induces the release of volatile organic compounds (VOCs) similar to those induced by herbivores in plants. Therefore, it has been used as a method to estimate attraction to VOCs in arthropod and avian predators. In this study, we examined whether potential differences in the composition of VOCs produced by herbivore-induced and MeJA-treated Pyrenean oak trees (*Quercus pyrenaica*) were related to differential avian attraction. Results showed that the overall emission of volatiles produced by MeJA-treated and herbivore-induced trees did not differ and were higher than emissions of Control trees. However, MeJA-treated trees seem to exhibit a higher reaction and release several specific compounds, which may explain the lack of avian attraction to MeJA-treated trees observed in some previous studies.

Abstract

The tri-trophic interactions between plants, insects, and insect predators and parasitoids are often mediated by chemical cues. The attraction to herbivore-induced Plant Volatiles (HIPVs) has been well documented for arthropod predators and parasitoids, and more recently for insectivorous birds. The attraction to plant volatiles induced by the exogenous application of methyl jasmonate (MeJA), a phytohormone typically produced in response to an attack of chewing herbivores, has provided controversial results both in arthropod and avian predators. In this study, we examined whether potential differences in the composition of bouquets of volatiles produced by herbivore-induced and MeJA-treated Pyrenean oak trees (*Quercus pyrenaica*) were related to differential avian attraction, as results from a previous study suggested. Results showed that the overall emission of volatiles produced by MeJA-treated and herbivore-induced trees did not differ, and were higher than emissions of Control trees, although MeJA treatment showed a more significant reaction and released several specific compounds in contrast to herbivore-induced trees. These slight yet significant differences in the volatile composition may explain why avian predators were not so attracted to MeJA-treated trees, as observed in a previous study in this plant-herbivore system. Unfortunately, the lack of avian visits to the experimental trees in the current study did not allow us to confirm this result and points out the need to perform more robust predator studies.

Keywords: avian olfaction; foraging; herbivore-induced plant volatiles; defense against herbivory

Introduction

The tri-trophic interactions between plants, insects, and insect predators and parasitoids are a dominant component of many terrestrial ecosystems [1]. As such, they are of particular interest to ecologists and are widely studied [1]. Within the tri-trophic interactions, many predators of herbivorous arthropods use chemical signals provided by the host plants when searching for insect prey [1,2]. Plants naturally contain large amounts of stored constitutive volatile organic compounds (VOCs), and these might be volatilized into the atmosphere by a healthy, unwounded plant depending on their physiochemical properties [3]. However, they are typically volatilized in greater qualities or quantities upon mechanical tissue breakage during herbivore attack [4]. Additional synthesis of novel compounds may be induced by elicitors contained in the saliva of herbivores [5]. After contact with herbivore-specific saliva, the plant synthesizes a hormone methyl jasmonate (MeJA) which mediates the release of so-called herbivore-induced volatile compounds (HIPVs) [6,7]. Induced VOCs may be emitted hours or days after an attack, both from the wounding site only or systemically from undamaged plant leaves [8–11].

Some of these HIPVs can act indirectly as attractants of natural enemies of herbivorous arthropods (e.g., [12–15]). Insectivorous predators can detect the volatile compounds, track the damaged plant, feed on the arthropods causing the damage, and reduce the abundance of herbivorous insects, thus enhancing the plant's fitness [16–18]. The attraction to HIPVs has been well documented for arthropod predators and parasitoids (predatory mites, parasitoid wasps, predatory bugs, nematodes, etc.—see [1,19,20] for reviews).

Mäntylä and collaborators (2004 [21]) suggested that also other critical predators, insectivorous birds, might be a part of this so-called Cry for Help hypothesis [22]. The authors found that birds discriminate between chemically active and intact trees without seeing the herbivorous larvae or the damage on the leaves [21]. Further, they proposed that the mechanism responsible for the attraction of birds to herbivore-induced trees could be vision [23] or olfaction [24], as herbivore-induced trees differed from uninfested trees both in the reflectance of leaves and the emission of HIPVs [23,25,26]. Subsequent research by Mäntylä and collaborators (2008 [24]) found a positive correlation between avian predation rates of artificial larvae and the quantity of volatiles emitted by mountain birches. Thus, suggesting that olfaction may be the mechanism underlying bird attraction to caterpillar-infested trees [24]. Later, Amo and collaborators (2013 [25]) isolated the chemical and visual cues of trees, and they showed that insectivorous birds safely preferred trees providing a chemical signal over the trees providing only visual signal [25]. Therefore, it seems that olfaction is used by birds to search for food [25,27], although the importance of vision and olfaction in the foraging behavior of birds is not completely understood yet [27,28]. Recent evidence shows that insectivorous birds can detect even small concentrations of HIPVs of herbivore-induced trees that are just developing new leaves [29], or small amounts of volatiles emitted during insect egg deposition [30]. Attraction to caterpillar-infested trees has been studied in different plant-insect-bird systems using ununified methodology [21,23,24,29–32] and often showing contradictory results.

The exogenous application of jasmonates, such as MeJA, often induces the release of volatile organic compounds (VOCs) similar to those

induced by herbivores in plants [6,11,33,34]. It has been proven that arthropod predators are attracted to MeJA-treated plants, as they are similarly attracted to plants infested by herbivores [2,6,11]. The use of MeJA to simulate insect herbivory has also been used as a method to study avian attraction to HIPVs but with less conclusive results. In the first study under natural conditions, Mäntylä and collaborators (2014 [35]) treated mature mountain birches (*Betula pubescens* Ehrh. Ssp. *Czerepanovii*) with different concentrations of MeJA solutions, and they found that insectivorous birds were not attracted to MeJA-treated trees while they were attracted to caterpillar-infested trees. Later, Saavedra and Amo (2018 [36]) reported that birds were not attracted to MeJA treated Pyrenean oaks (*Quercus pyrenaica* Willd 1805). In contrast, three recent studies showed that wild, insectivorous birds increased their affinity to MeJA-treated mature grey willow shrubs (*Salix cinerea*) [31], English oak *Quercus robur* [11], and *Ficus hahliana* trees [37].

Due to the inconsistency of previous results, further research is needed to examine the bird attraction to MeJA-treated trees in different plant-herbivore interactions and to disentangle the causes of the variation in the response of birds to the exogenous application of MeJA. Specifically, it is necessary to determine whether the volatiles released by MeJA-treated trees are similar to those emitted by herbivore-infested trees, as potential differences in the volatile emission between herbivore-infested trees and MeJA-treated trees may explain such differences regarding avian attraction to MeJA-treated trees.

As mentioned earlier, Saavedra & Amo (2018 [36]) found that 5 mM MeJA-treated Oak trees (*Quercus pyrenaica*) were slightly more frequently visited than untreated trees, but differences were not significant.

Unfortunately, whether volatiles emitted by MeJA-treated trees differed from those emitted by herbivore-induced trees was not examined at that time. Here, we present the results of a study aimed to study insectivorous bird attraction to Pyrenean oak trees treated with MeJA and to herbivore-induced trees, and to find the mechanisms underlying avian attraction. We hypothesized that the lack of interest of the birds in MeJA-treated trees in the earlier study [36] could be explained by the lack of similarity of the volatiles emitted by herbivore-induced trees and MeJA-treated trees.

Materials and methods

Study Area and Species

The experimental study was carried out in May 2021 in a Pyrenean oak (*Quercus pyrenaica*) forest in Madrid province (Sierra de Guadarrama, Central Spain, 40°43' N, 03°55'W). In this forest, a population of insectivorous birds breeding in 100 wooden nest-boxes was established in 2017. Nest boxes were occupied mainly by breeding pairs of blue tits (*Cyanistes caeruleus*), and fewer pairs of great tits (*Parus major*). Other insectivorous bird species were observed in the study area at lower densities, including the common blackbird (*Turdus merula*), coal tit (*Periparus ater*), and Eurasian nuthatch (*Sitta europaea*). Tits feed mainly on caterpillars, such as the *Operopthera brumata* or *Tortrix viridiana*, during the breeding period in this region [38,39]. Previous monitoring showed that *T. viridiana* was more abundant on Pyrenean oaks than *O. brumata* (I. Saavedra, personal observation). Therefore, we selected *T. viridiana* as the prey and model species. One week before the experimental study, we hand-collected wild *T. viridiana* caterpillars from oak leaves in the forest and kept them in captivity until they were 5th instar stage.

Caterpillars were located in 7×6 cm polypropylene containers and fed with fresh leaves of *Quercus pyrenaica*. Water was provided via daily spraying of the leaves.

Experimental Design

We selected 45 adult Pyrenean oak trees with trunks of at least 20 cm in diameter at breast height that were separated by at least 20 m. The experimental trees were alternatively assigned to one of the treatments: MeJA-treated trees ($n = 15$), herbivore-induced trees ($n = 15$), and control trees ($n = 15$). At each tree, we selected one focal branch to which we applied the following treatments. The branches were approximately 0.5–1 m long and 1.5 m above the ground and had no evident signs of herbivory.

The MeJA treatment consists of applying a 5 mM solution made with distilled water, ethanol, MeJA, and Tween–20. We chose a 5 mM dose as the results of the previous study showed that more insectivorous birds visited the oak trees treated with this MeJA solution than the control and 15 mM MeJA solution treated trees, although differences were not significant [36]. The MeJA-treated trees were prepared by spraying 10 ml of the MeJA solution on a bouquet of leaves (7–10 leaves) on the focal branch of each tree. Each focal branch had around 5–7 bouquets of 7–10 leaves per bouquet, meaning the treatments were applied to 14–20% of the leaves of the focal branch. The treatment was applied every two days for 14 days (i.e., 7 times).

Herbivore-induced trees were prepared by placing 10 individual *T. viridiana* caterpillars on a bouquet of leaves on the focal branch and placed into a green organza sachet (20×20 cm) covering the bouquet of leaves (Figure 1). Caterpillars were kept inside the sachet for an entire 14 days. At

the end of the experiment, the organza sachet was removed and the caterpillars were released to move to other branches.

Similar empty organza sachets were installed at the rear bouquets of leaves of the focal branches of MeJA and Control treatments to control the effect of the sachet itself (Figure 1). Additionally, control and herbivore-induced trees were sprayed with 10 mL of distilled water according to the schedule of MeJA treatment to avoid differences in the appearance of the moist leaves.

To study the attraction of the insectivorous birds to the trees, we measured the predation rate of birds on artificial larvae. We placed 5 artificial larvae on branches of each tree. The artificial larvae were made of light green plasticine (similar to the natural color of real *T. viridiana* caterpillars, Lepidoptera, Tortricidae, at least according to human-visual perception). The plasticine larvae were similar in size to large fifth instar *T. Viridiana* caterpillars (length 15–20 mm, diameter 3–4 mm). The plasticine larvae were attached with cyanoacrylate adhesive glue to the branches of each tree.

We measured the attraction of birds to each tree by checking the number of plasticine larvae with marks indicating predation by birds. This method has been used in previous studies of avian predation [24,31,35,36,40–46]. The artificial larvae are considered damaged when they had triangle-shaped marks and deep cuts made by bird beaks and when a part of their body was taken by the birds (see [24,35]). Each larva showing a predation mark was replaced with a new one at the same location when larvae were checked during check visits.

The experimental study was conducted under a license issued by the Dirección General de Biodiversidad y Recursos Naturales, Consejería de

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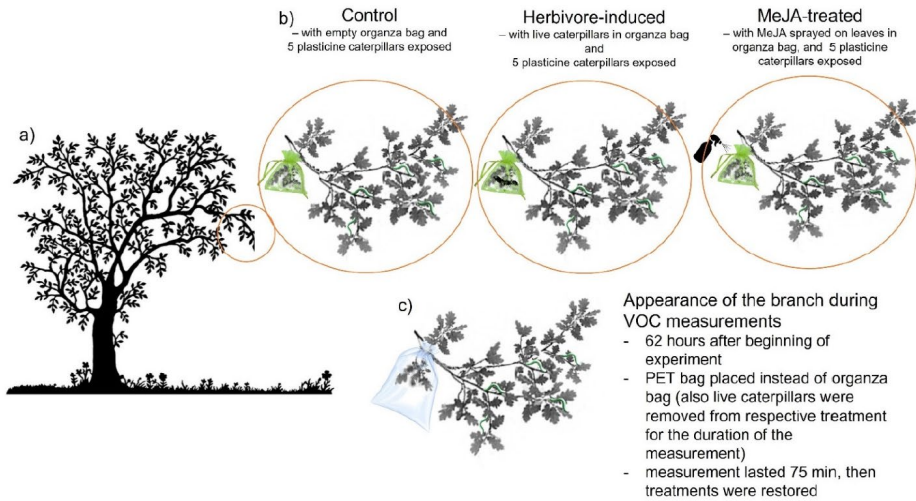


Figure 1. Experiment design showing the approximate location of an experimental branch at each of the focal trees (a), three respective treatments as they looked like in the 14 days long predation experiment - Control, herbivore-induced, MeJA-treated (b), and appearance of the branches during the measurement of volatile compounds (c). The measurement started 62 h after the beginning of the experiment, upon removal of the organza bag and real caterpillars from herbivore-induced treatment. Volatiles were then collected for 75 min. After that, the organza bag and live caterpillars were returned to their respective treatments. For the MeJA application (b), the organza bag was always removed, MeJA sprayed on the leaves inside, and the bag returned to the branch.

Collection of Plant Volatiles

We collected plant volatiles of all experimental trees ($n = 45$), and four additional blank samples, 62 h (mean \pm SE = 62 ± 1.05 h) after the beginning of the experiment, i.e., after placing caterpillars on the sachets or applying MeJA treatment. We collected volatiles 62 h after adding the treatments because previous results in another *Quercus* species, the downy oak tree (*Quercus pubescens* Willd), suggest that HIPVs significantly increase from 48 h after infestation by winter moth (*Operophtera brumata*)

and green oak tortrix (*Tortrix viridana*) when compared to uninfected control trees (Graham et al. unpublished data, [29]). For each volatile measurement, we removed the organza bag from each branch and removed the caterpillars from the Herbivore-treated trees. We placed the bouquet of leaves where treatments were applied (i.e., MeJA was sprayed and caterpillars present) into a polyethylene terephthalate (PET) bag (Carrefour®) closed with a tap. We kept the bag for 60 min, passively filling it with volatiles. Then, we cut one corner of the bag, put in a glass tube for thermal desorption (TD) containing approximately 100 mg of Tenax TA adsorbent (Supelco, mesh 60/80; Bellefonte, PA, USA). The TD tube was connected to a vacuum pump (PAS-500, Spectrex, Redwood City, CA, USA) through a silicon tube. The air was pulled through the TD tube at a flow rate of 200 mL/min. An active volatile sampling was conducted for 15 min, and a total of 3 l of air was pulled through each tube. After that, the TD tubes were removed, closed with silicon caps, and kept refrigerated at 4 °C for about 1 week before analysis by gas chromatography-mass spectrometry. We also measured four blank VOC profiles from empty bags (i.e., background emissions). We sampled the volatiles on two sunny days, between 10:00 and 18:00, with a similar mean temperature (mean \pm SE= 25.22 \pm 0.59) and humidity (mean \pm SE= 46.16 \pm 1.04) during sampling. Therefore, there were no significant differences between treatments in the temperature (ANOVA, $F_{2,42} = 0.03$, $p = 0.97$) or relative humidity (ANOVA, $F_{2,42} = 0.01$, $p = 0.99$) during sampling volatiles, nor in the time the bag was covering the branch (ANOVA, $F_{2,42} = 0.00$, $p = 1.00$).

Analysis of Plant Volatiles

Before the experiment, TD tubes were conditioned with a gas chromatograph (5890 Agilent, modified for spike and cleaning TD tubes)

for 30 min at 320 °C and a Helium flow rate of 20 mL/min at the Institute of Environmental Technology in Ostrava-Poruba, Czech Republic. The induced volatile samples were analyzed with a gas chromatograph–mass spectrometer (Agilent, GC 7890 + MSD) at the Institute of Environmental Technology in Ostrava-Poruba, Czech Republic. Trapped compounds were desorbed with two-stage thermal desorption using a thermal desorption unit (Perkin- Elmer Turboamatrix TD 300) under described temperatures: Valve = 200 °C, Primary desorption = 250 °C 5 min⁻¹, Trap = -10 °C, Secondary desorption = 300 °C min⁻¹, Transferline = 200 °C; and flows: Desorption = 40 mL min⁻¹, Intel Split = 0 mL min⁻¹, Col. = 1 mL min⁻¹, Out Split = 10 mL min⁻¹; Total split = 9.1%).

Desorbed analytes were injected onto an HP-5 capillary column (30 m, 0.25 mm, 0.25 µm film thickness, Hewlett-Packard) with helium (5N) as a carrier gas with a flow rate of 1 ml min⁻¹. The oven temperature program was held at 60 °C for 2 min, then raised to 120 °C at a rate of 10 °C min⁻¹, and finally on to 250 °C at a rate of 30 °C min⁻¹ with a 5 min delay. The compounds (mono-, homo- and sesquiterpenes, and green leaf volatiles (GLVs)) were identified by comparing their mass spectra with those in the pure standards. Pure chemicals were supplied by Sigma-Aldrich and Supleco, prepared by weighing into methanol:

- (1) Cannabis terpene Mix B (CRM40937 Supleco, 2000 µg/mL of each component): Limonene (cyclohexene, 1-methyl-4-(1-methylethenyl)-), C₁₀H₁₆, CAS 138-86-3; β-pinene (bicyclo[3.1.1]heptane-6,6- trimethyl, 2-methylene), C₁₀H₁₆, CAS 127-91-3; β-Caryophyllene (trans-(1R,9S)-8-Methylene-4,11,11-trimethylbicyclo[7.2.0]undec-4-ene), C₁₅H₂₄, CAS 87-44-5; Phytol (3,7,11,15-Tetramethyl-2-hexadecen-1-ol),

$C_{20}H_{39}O$, CAS 7541-49-3; Geraniol (trans-3,7-Dimethyl-2,6-octadien-1-ol), $C_{10}H_{18}O$, CAS 106-24-1; (1S)-(-)-Camphor ((1S)-1,7,7-Trimethylbicyclo[2.2.1]heptan-2-one), $C_{10}H_{16}O$, CAS 464-48-2; Terpinolene (p-Menth-1,4(8)-diene), $C_{10}H_{16}$, CAS 586-62-9; β -Eudesmol ((2R,4aR,8aS)-Decahydro-8-methylene- $\alpha,\alpha,4a$ -trimethyl-2-naphthylmethanol), CAS 473-15-4; (+)-Borneol (endo-(1R)-1,7,7-Trimethylbicyclo[2.2.1]heptan-2-ol), $C_{10}H_{18}O$, CAS 464-43-7; cis-Nerolidol (3,7,11-Trimethyl-1,6,10-dodecatrien-3-ol), $C_{15}H_{26}O$, CAS 7212-44-4; α -Terpineol (2-(4-Methylcyclohex-3-en-1-yl)propan-2-ol), $C_{10}H_{18}O$, CAS 98-55-5; (1S)-(+)-3-Carene ((1S)-3,7,7-Trimethylbicyclo[4.1.0]hept-3-ene), $C_{10}H_{16}$, CAS 498-15-7; Linalool ((\pm)-3,7-Dimethyl-3-hydroxy-1,6-octadiene), $C_{10}H_{18}O$, CAS 76-70-6; p-Cymene (1-Isopropyl-4-methylbenzene), $C_{10}H_{14}$, CAS 99-87-6.

(2) Cannabis terpene Mix A (CRM40755 Supleco, 2000 $\mu\text{g/mL}$ of each component): α -Pinene (2,6,6-Trimethylbicyclo[3.1.1]hept-2-ene), $C_{10}H_{16}$, CAS 80-56-8; Camphene (3-methylidenebicyclo[2.2.1]heptane), $C_{10}H_{16}$, CAS 79-92-5; β -Myrcene (7-Methyl-3-methylideneocta-1,6-diene), $C_{10}H_{16}$, CAS 12-35-3; 3-Carene (3,7,7-Trimethylbicyclo[4.1.0]hept-3-ene), $C_{10}H_{16}$, CAS 13466-78-9; D-Limonene (1-Methyl-4-(prop-1-en-2-yl)cyclohex-1-ene), $C_{10}H_{16}$, CAS 5989-27-5.

(3) Single chemicals (Sigma-Aldrich): Caryophyllene oxide, CAS 1139-30-6; Ocimene (3,7-Dimethyl-1,3,6-octatrien), $C_{10}H_{16}$, CAS 13877-91-3; cis-3-hexenyl Acetate, $C_8H_{14}O_2$, CAS 3681-

71-8; Methyl Jasmonate (Methyl 3-oxo-2-(2-pentenyl) cyclopentaneacetate), $C_{13}H_{20}O_3$, CAS 39924-52-2. Emissions were presented qualitatively.

Statistical Analyses

We used STATISTICA 8.0 to perform one-way ANOVA to analyze whether there were significant differences between treatments in the time the bag was covering the branch before measuring volatiles, as well as in the temperature and relative humidity (RH).

We used program R (version 4.0.0; [47]) and *MASS* package [48] to analyze the effect of treatment (MeJA-treated, herbivore-induced, Control) on the total amount of emitted VOCs fitting a Linear Model (package *stats*; [47]). The response variable (total amount of emitted volatiles) was computed by natural logarithm ('log' function). For analyses of the effect of treatment (MeJA-treated, herbivore-induced, Control) on individual compounds mean change emission, a Generalized Linear Mixed Models with Template Model Builder (package *glmmTMB*; [49]) was fitted. The Estimated marginal means and multiple contrasts among factors were made using the package *emmeans* [50].

Results

We did not find any plasticine caterpillar with avian predation marks in any of the three treatments, despite exposing a total of 225 of them for 14 days. This prevented any further analyses of differences in predation between treatments and its link to the chemistry of branches.

Analyses of volatile compounds detected more than 100 different chemicals of various types. We focused on 17 terpenoids differing considerably between the treatments in detail. The amounts of terpenoids

released by herbivore-induced and MeJA-treated trees were significantly higher than terpenoid emissions of control trees (ANOVA $\text{Chisq} = 21.374$, $\text{Df} = 2$, $p < 0.001$ [Figure 2]). Total emissions of HIPVs released by MeJA-treated and herbivore-induced oaks did not differ significantly ($p = 0.53$).

Specifically, the application of MeJA resulted in significantly higher production of Trans- β -Ocimene ($p < 0.05$) and Bourbonene ($p < 0.001$) compared to the chemical emissions of control trees (Figure 3). A marginal significance of increased production of β -Ocimene ($p = 0.054$) was also detected. For herbivore-induced trees, the amount of Bourbonene ($p < 0.001$) was significantly different from those emitted from control trees (Figure 3). Within the surveyed compounds, we found only one qualitative difference. MeJA-treated trees did not produce Isolongifolen, which was produced both by herbivore-induced and control trees.

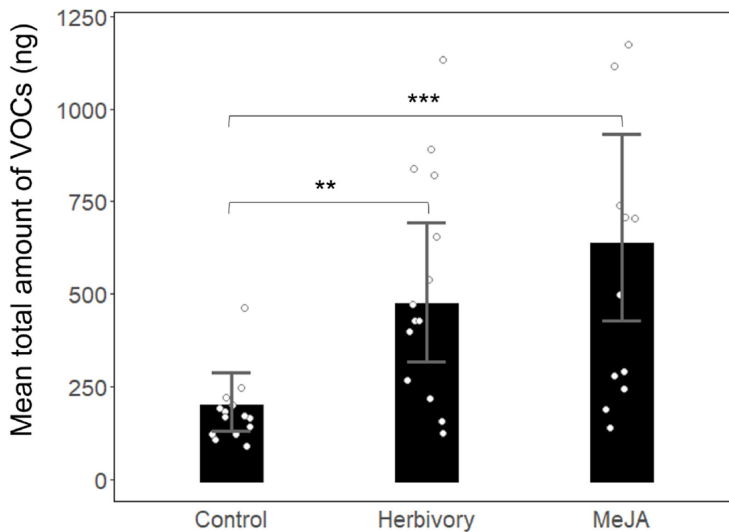


Figure 2. The total amount of volatiles (ng) emitted by Control ($n = 15$), herbivore-induced ($n = 15$), and MeJA-treated ($n = 15$) Pyrenean oaks at a study site in an oak forest of Guadarrama Mountains, Spain. Treatments with significantly different amounts of volatiles are marked by asterisks (***) $p < 0.001$, ** $p < 0.01$); results of chisq test. White circles show VOCs emitted by each individual tree.

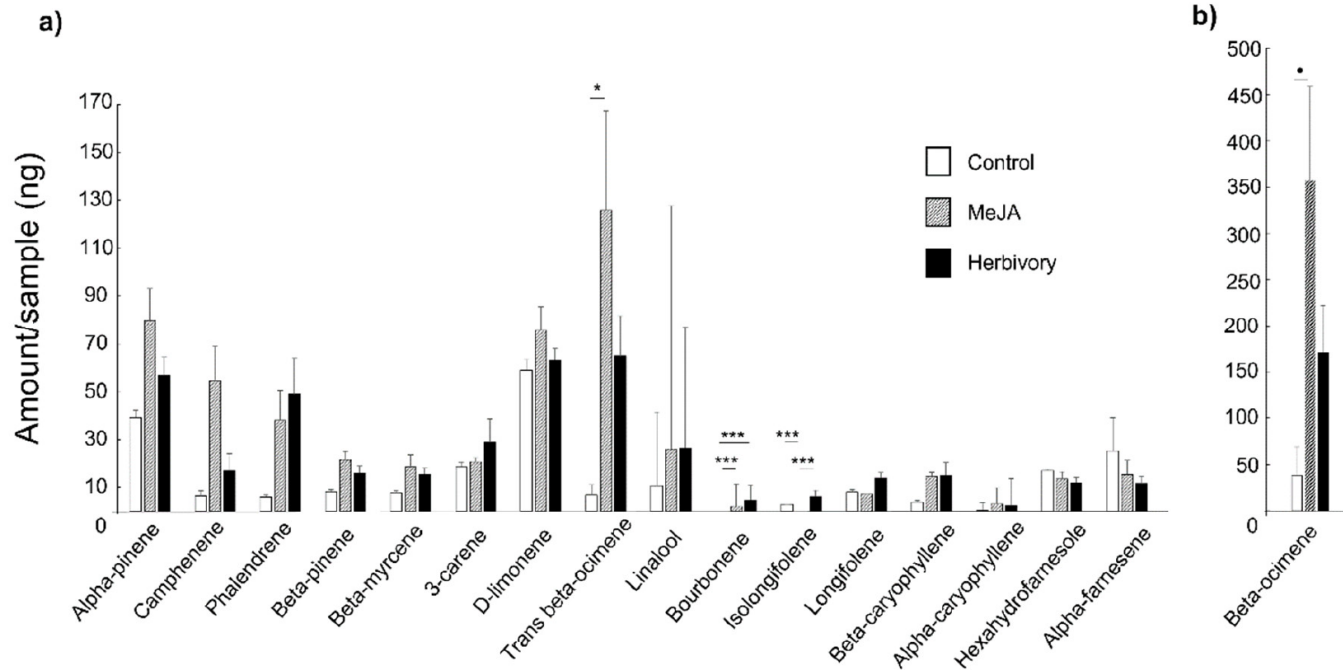


Figure 3. Mean change in the VOCs emission between MeJA-treated, herbivore-induced, and Control trees of Pyrenean oak. The x-axes show the individual compounds, and y-axes show the relative amount (ng; in **a** and **b** with different y-axis scales) of emitted VOCs. MeJA-treated and herbivore-induced trees released significantly higher amounts of Bourbonene (**a**) compared to control trees. MeJA-treated trees emitted more Trans- β -Ocimene and β -Ocimene (**b**) than the control trees. MeJA-treated trees did not produce Isolongifolen in contrast to the control and herbivory-infested trees (**a**). Treatments with significantly different amounts of volatiles are marked by asterisks (***) $p < 0.001$, * $p < 0.05$); results of chisq test.

Discussion

Our results suggest that the overall emission of VOCs produced by MeJA-treated and herbivore-induced trees did not differ, and both were rather different from the emissions of control branches. However, MeJA treatment showed a stronger reaction of experimental branches, as they released several specific compounds in contrast to herbivore-induced trees. Thus, the application of MeJA in manipulative experiments might have caused the overreaction of studied plants and influenced the potential reactions of predators. An alternative explanation is that we did not correctly select the dose of MeJA to match the HIPVs induced by the selected number of caterpillars we used on infest trees.

In a previous study examining avian attraction to MeJA-treated oak trees, results showed that more birds visited the oak trees treated with 5 mM MeJA (7/11) than untreated control trees (4/11) [36]. However, differences were not significant in this previous study, perhaps because this study had low power to detect differences in bird attraction between treatments [36]. To disentangle the questions pointed out in the previous study, we decided to replicate the study with an increased sample size and with adding a new treatment, i.e., herbivore-induced trees. However, in the current study we had an unexpected result, as we did not find any caterpillars being attacked by avian predators. The use of similar artificial larvae for estimating bird attraction to trees has been used successfully in several studies [24,31,36,40–45], even within the same bird population [46]. Therefore, the lack of attraction even to Herbivore-infected trees is unlikely due to the use of artificial caterpillars. Another reason might be that the presence of the green organza sachet on the branches scared birds approaching the experimental branches. However, previous studies also used similar sachets or bags to

keep caterpillars on a branch in field experiments [24,35] and found birds attacking artificial caterpillars even close to the bags. Therefore, the use of sachets does not seem to be a methodological artifact that may have masked bird attraction to, at least, herbivore-infected trees. A third possible explanation could be that the bird species present in the study area may not discriminate between trees emitting herbivore-induced volatiles or uninfested trees. However, blue tits and great tits are the most abundant insectivorous bird species in the study area, so this explanation can be discarded as both species are already known to be attracted to herbivore-infested trees ([23–25,30,51], but see [52]).

Another possible factor impacting our results could be we only treated or infested a small bouquet of leaves on each large experimental tree. It is known that the induced VOCs may be emitted from both the specific place of herbivorous wounding or MeJA application, or systemically from undamaged leaves [8,10,11,53–58]. As we measured volatiles from the leaves that were directly herbivore-induced or MeJA-treated, these exact leaves were not accessible to birds because they were inside the sachets. To our knowledge, there are no studies that have examined how localized the response to MeJA exogenous application in *Quercus pyrenaica* is, but the results of a very recent study with a close species, *Quercus robur*, showed that the control branches close to the MeJA-treated branches did not produce more VOCs; i.e., this plant species exhibits a highly localized response [11]. Assuming that the response to MeJA is similarly localized in *Quercus pyrenaica*, it may be that the birds were not able to detect it within the bags. Finally, another possible and nonexclusive explanation of the lack of attraction to herbivore-infested trees is the high availability of food in the forest. We performed the experiment during the spring of an especially rainy

year when there was an unusually high abundance of insect prey (I. Saavedra and L. Amo, personal observation).

Despite the fact we could not obtain bird behavioral data in this study, our results from chemical analyses suggest that the slight differences in the emission of volatiles may explain the lack of significant differences in the attraction of insectivorous birds to MeJA-treated and control Pyrenean oak trees found in a previous study in this plant-herbivore-bird system [36]. Combined results of our current and earlier study [36] are in line with results of a study where mature mountain wild birches (*Betula pubescens* ssp. *czerepanovii*) were treated with 15 mM and 30 mM MeJA solutions and infested with larvae of the autumnal moth (*Epirrita autumnata*) in field conditions. The authors found a significant difference in chemical response to treatment but did not detect a significant difference in attractivity of the MeJA-treated trees in comparison to the control. In both studies, (current and [35]), increased production of α -pinene, both on herbivore-induced and MeJA-sprayed trees, and increased production of limonene and myrcene from MeJA-treated trees was detected.

In contrast, MeJA-treated plants attracted insectivorous birds and predatory arthropods in three studies performed in different systems, using grey willows *Salix cinerea* in the Czech Republic [31], MeJA-treated *Ficus hahliana* in Papua New Guinea [32], and *Quercus robur*, *Carpinus betulus*, and *Tilia cordata* in Germany [11]. Although volatiles were not determined in *Ficus hahliana* trees, Mrazova & Sam [37] measured the HIPVs of grey willows treated with 30 mM MeJA solution and untreated shrubs. The production of α -pinene, β -pinene, 3-carene, limonene, and β -ocimene was higher in MeJA-treated shrubs than in untreated shrubs [31]. In *Quercus robur* and *Carpinus betulus* trans- β -ocimene increased significantly after

MeJA treatment in contrast to the control treatment, but there was no significant effect of MeJA on any compound found in *Tilia cordata*. Unfortunately, herbivore-induced trees were not included in any of the three above-mentioned studies, so it is impossible to say whether herbivore-damaged trees and MeJA-treated trees would differ in their volatile emission and how this could affect avian attraction.

Many studies focusing on arthropod predators have also found differences between the volatiles emitted by plants in response to exposure to jasmonate and those emitted by herbivore-infested plants [34,59–61]. For example, MeJA-treated lima bean plants released similar but not identical HIPVs as those released by herbivore-infested plants [62,63]. Although MeJA-treated lima bean plants were still attractive to predatory mites, spider-mite-infested plants were preferred by predatory mites [59]. In contrast, other studies found an attraction of arthropod predators or parasitoids to jasmonate-treated plants (e.g., [7,11,64–66]). Differences in the volatile emission between MeJA-treated and herbivore-induced trees can be expected because plant defense responses display a great deal of specificity [67] despite jasmonate representing an important mediator of chemical defense in plants, especially in response to lepidopteran caterpillar herbivory [68]. As a mediator, MeJA is expected to elicit only a more generalized response than the damage caused by any specific herbivore [69]. Our results seem to support it being in line with this expectation as MeJA-treated trees emitted more Trans- β -Ocimene and β -Ocimene than control trees, but no more than herbivore-induced trees. Furthermore, we found that MeJA-treated trees did not produce Isolongifolen in contrast with Control and herbivore-induced trees. Previous results also found that different plant species respond to MeJA treatment by emitting several VOCs, some of

which were detected in the herbivore-wounded plants [11], but others were unique to the MeJA treatment [34]. Further experimental studies are needed to disentangle which compounds, and their relative proportions, of these volatile blends can have an important attractive or deterrent role in the discrimination herbivoreinfected and uninfected trees.

The variability of HIPVs emissions can be further determined by other factors [19,70]. For example, the emission of HIPVs is known to differ according to the plant species [71,72], the developmental stage of the plant [73], and even the parts attacked by the herbivores [74]. For example, in a previous study with northern red oak (*Quercus rubra*) seedlings, the authors found that the activity of peroxidase isozymes are involved in the tree defense response differed between seedlings treated with MeJA and those infected with caterpillars [75]. The emission of HIPVs also depends on the arthropod species [22,72,76], on the herbivore density [77–79], and even on the time course after infestation [80]. Moreover, environmental factors are also known to influence the emission of HIPVs. For example, differences in HIPVs have been found between laboratory and field conditions [6,81]. All of these factors can induce quantitative or quality changes in the volatile blend [82–84]. Furthermore, differences in the dosage of jasmonate may also influence the release of volatiles, as well as predator or parasitoid attraction [85]. We decided to use a dose of 5 mM MeJA, as previous results suggested that birds visited oaks trees treated with this dose more often than oaks treated with a higher dose [36]. Further, necrosis on the leaves of the trees treated with 15 mM MeJA solution was observed. However, we have to admit that we do not know how exactly this dose mimics the situation when oak trees are infected by caterpillars. Furthermore, based on previous results with *Quercus pubescens*, where authors found that the emission of VOCs

significantly increased from 42 h after infestation (Graham et al. unpublished data, in [29]), we decided to measure the volatiles of our trees after 62 h from infestation or MeJA application. Therefore, differences in the dosage as well as in the timing of the measurement may have provided different results in the analysis of volatiles.

Insectivorous birds are generalist predators that feed on different prey species hosted by different plant species. For them, the signal of the presence of insects should be sufficiently generalistic, yet significantly different from the emissions of VOCs released after simple mechanical wounding. As the jasmonic pathway is involved in both processes, the ability to learn to associate a positive foraging experience with the particular blends of HIPVs seems to be favored over an innate recognition of so many different blends of volatiles ([15,51,86], but see [87]). This ability to associate HIPVs with the presence of food, as well as an innate lack of attraction to infested trees, has been previously demonstrated in two experimental studies with naïve great tits [51,88]. Great tits naïve to foraging in trees were not attracted to herbivore-induced trees, whereas when they experienced foraging experiences, they were able to discriminate between the volatiles of herbivore-infected and uninfected trees, both from native [51,88] and foreign trees species [51]. Therefore, the lack of attraction to MeJA-treated oak trees found in a previous experiment [36] can be explained by the inability of experienced wild birds to associate the volatile blend of MeJA-treated oak trees with any blend of HIPVs Pyrenean oak trees infested with herbivorous prey.

Conclusions

Overall emission of VOCs produced by MeJA-treated and herbivore-induced trees did not significantly differ from each other. However, MeJA treatment seems to cause a more significant reaction of experimental trees related to the release of several specific compounds compared to herbivore-induced trees. Yet, blends from these two treatments differed from VOCs of Control trees. Whether these slight differences in the emission of volatiles between trees influenced insectivorous bird attraction to MeJA-treated trees remains to be explored. Furthermore, research consensus about the dosage of MeJA, minimal and suitable number of treated leaves per tree, as well as the timing of the volatile measurement after treatment application or infestation should be reached in order to have comparable data among the different studies. The lack of attraction to MeJA-treated and herbivore-induced trees in our study also points out the potential need to perform the experimental studies in periods of low prey availability, or conduct much larger experiments, to increase the relative chances of caterpillars being attacked.

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Chapter III

Exogenous application of methyl jasmonate to *Ficus hahliana* attracts predators of insects along an altitudinal gradient in Papua New Guinea.

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**Exogenous application of methyl jasmonate to
Ficus hahliana attracts predators of insects along
an altitudinal gradient in Papua New Guinea.**

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Abstract

In many plants, the defence systems against herbivores are induced, and may be involved in recruiting the natural enemies of herbivores. We used methyl jasmonate, a well-known inducer of plant defence responses, to manipulate the chemistry of *Ficus hahliana* along a tropical altitudinal gradient in order to test its ability to attract the enemies of herbivores. We examined whether chemical signals from MeJA-treated trees (simulating leaf damage by herbivores) attracted insect enemies in the complex settings of a tropical forest; and how this ability changes with altitude, where the communities of predators differ naturally. We conducted the research at four study sites (200, 700, 1700 and 2700 m asl) of Mt Wilhelm in Papua New Guinea. Using dummy plasticine caterpillars to assess predation on herbivorous insect, we showed that, on average, inducing plant defences with jasmonic acid in this tropical forest increases predation twofold (i.e. caterpillars exposed on MeJA-sprayed trees were attacked twice as often as caterpillars exposed on control trees). The predation rate on control trees decreased with increasing altitude from 20.2% d⁻¹ at 200 m asl to 4.7% d⁻¹ at 2700 m asl. Predation on MeJA-treated trees peaked at 700 m (52.3% d⁻¹) and decreased to 20.8% d⁻¹ at 2700 m asl. Arthropod predators (i.e. ants and wasps) caused relatively more attacks in the lowlands (200–700 m asl), while birds became the dominant predators above 1700 m asl. The predation pressure from birds and arthropods corresponded with their relative abundances, but not with their species richness. Our study found a connection between chemically induced defence in plants and their attractivity to predators of herbivorous insect in the tropics.

Chapter III

Keywords: Herbivore-induced plant volatile, HIPV, methyl jasmonate, multitrophic interactions, plasticine caterpillars, predators, tropical gradient

Introduction

The understanding of communication pathways is of particular interest in the context of complex tritrophic interactions between plants, insects and insect predators and parasitoids (Heil 2014) because interactions between plants and their arthropod herbivores and their natural enemies dominate the terrestrial ecology of our planet. It has been shown that, in nature, many predators of herbivorous insects are attracted to their prey through signals provided by the host plants (Heil 2014, Zhang et al. 2009). The attraction between host plants and insect predators is often mediated by volatile organic compounds (VOCs) (De Moraes et al. 1998, Turlings et al. 1990), particularly those induced by plants which have been damaged by herbivores (i.e. herbivore-induced plant volatiles, HIPVs). Mäntylä et al. 2004) were the first to suggest that insectivorous birds may also use HIPVs to search for prey on plants. The attractiveness of herbivore-damaged trees to birds was later tested in both aviaries (Amo et al. 2013, Koski et al. 2015, Mäntylä et al. 2004, 2008) and nature (Koski et al. 2015, Mäntylä et al. 2008). Only seven out of 10 existing studies have shown that birds significantly distinguish trees with and without herbivore damage (either natural or induced/simulated chemically). Amo et al. (2013) advanced this field by showing that when birds were provided separately with a chemical and/or visual signal they relied solely on olfaction.

Jasmonates, i.e. jasmonic acid (JA) and methyl jasmonate (MeJA), are endogenous plant phytohormones that regulate a large number of defence responses in plants (Hopke et al. 1994, Rodriguez-Saona et al. 2013, Thaler et al. 1996, Xu et al. 2003). Plants treated with exogenous JA or MeJA were shown to emit volatiles similar to those given off by

plants attacked by live herbivores (Rodriguez-Saona et al. 2013). To our knowledge, there have only been three studies conducted using trees growing outdoors and none have been conducted under natural conditions in the complex forest systems of tropical regions, where pressure from insect predators and parasitoids is usually higher than in temperate regions (Jeanne 1979, Roslin et al. 2017, Schemske et al. 2009).

Both insectivorous birds and predatory arthropods (mostly ants) are important predators, and the overall predation of herbivorous insects is determined to a large extent by their combined effects (Mooney 2007). In many tropical regions, arthropod enemies are responsible for more predatory attacks than insectivorous birds (Roslin et al. 2017, Sam et al. 2015a), but their relative importance may differ between different tropical sites (Van Bael et al. 2003, 2008). However, their abundance and predation usually decrease towards the higher altitudes of the mountains (Roslin et al. 2017, Sam et al. 2015a). In the tropics, altitudinal trends in the strength of trophic interactions are poorly known, since most studies have focused on lowland forest (Novotny & Basset 2005), and there has not been a single study which has focused on the importance of plant-produced chemical signals for predators in the natural settings of a tropical forest.

The aim of this study was to investigate the dynamics of natural predation on herbivorous insects (represented by dummy caterpillars; Howe et al. 2009, Low et al. 2014, Sam et al. 2015b) in response to the application of MeJA to saplings in forest understorey in the natural conditions of an altitudinal gradient (200–2700 m asl), in Papua New Guinea. We hypothesized that (1) MeJA-simulated herbivory would lead to increased predation pressure on the dummy prey, and (2) that the

relative proportion of attacks, caused by different groups of predators (birds vs. arthropods), would differ along the altitudinal gradient.

Methods

Study sites

The experiment was conducted along the altitudinal gradient of Mt Wilhelm (05°48'0S, 145°02'0E) in the Central Range of Papua New Guinea at four study sites at 200, 700, 1700 and 2700 m asl. The gradient and its study sites are described in detail elsewhere (Sam et al. 2015a). We conducted the 4-wk-long experiment at the very beginning of the rainy season, in October 2015 (the dry season was extended until the end of September due to El Niño). The season corresponds with the usual period of increased leaf flush and caterpillar appearance.

Plant species

We selected the understorey species *Ficus hahliana* (Diels) as our focal tree species because it has a large altitudinal distribution (0–2700 m asl in Papua New Guinea; Segar et al. 2016), suitable leaf size. We established a 2250-m-long transect at each study site, along which we selected 24 accessible saplings which were at a distance of at least 60 m from each other and which had foliage between 2 and 3.5 m above the ground. This spacing ensured that the experimental trees could be considered independent, as in field conditions, the transmission of VOCs by air is known up to 60 cm. At longer distances, VOCs are known to degrade (Karban 2007). We only worked with saplings which appeared healthy, that had newly flushed leaves and lacked signs of extensive herbivore damage. All focal saplings, at each site, were marked with a small piece of flagging tape on the lower part of the trunk and numbered (1–24).

Every odd-numbered tree was considered a control tree and every even-numbered tree was treated with MeJA.

MeJA treatment

The MeJA-treated saplings were prepared by administering daily (for 5 d) morning sprayings with 25 ml of a 30 mM MeJA solution (diluted in distilled water; Sigma Aldrich 30 mM J2500 MeJA; Mrazova & Sam 2018). The control trees were not sprayed with distilled water, as the trees received nightly rainfall and their leaves were already wet at the time of the MeJA application. Twenty-four hours after the first application of MeJA, we exposed five artificial caterpillars (Howe et al. 2009, Tvardikova & Novotny 2012) on each experimental tree (both MeJA-treated and control) to assess the response of predators on herbivorous insects.

Artificial caterpillars

We prepared the artificial caterpillars (3 mm × 2.5 cm) by pressing modelling clay (dark green, oil-based and non-toxic, Koh-I-Noor Hardtmuth, Ceske Budejovice, Czech Republic) through a syringe to ensure a completely smooth surface. The artificial caterpillars mimicked a commonly occurring moth of genus *Choreutis* (Sam et al. 2015a). The method of using artificial caterpillars is suitable for studies that compare the attack rates in various habitats and treatments. It also allows for the coarse identification of predators as was required for our study (Posa et al. 2007, Sam et al. 2015a).

The artificial caterpillars were pinned to each MeJA-treated and control tree. They were pinned on to the distal half of the young leaves in such a way that the head of the pin was hidden in the modelling clay and

then passed through the leaf. On each sapling we approximated the natural density of two artificial caterpillars m^{-2} of leaf area, which is constant across all the studied sites (V. Novotny, unpubl. data). After 24 h, each caterpillar was inspected, attack marks were photographed, and predators were identified into rough categories of either arthropods or birds (Low et al. 2014). All missing caterpillars and those with attack marks were replaced with new ones after the 24-h period. All the new artificial caterpillars were pinned in different locations on the same sapling to decrease the possibility that the predators would learn to search for them or avoid them. The experiment ran for 4 d at each study site (i.e. caterpillars were replaced four times and MeJA was applied five times). The caterpillars which were attacked by two different types of predator ($N = 2$) within 1 d were treated as two independently attacked caterpillars.

Bird and ant sampling

We surveyed the bird communities by conducting point counts at each altitude. Point counts (15 min per point) were carried out between 5h45 and 11h00 at 16 points (radius = 50 m) regularly spaced along the 2250-m transect. We conducted three pointcount surveys (i.e. 16 points \times 15 min \times 3 days per each study site) during the experiments with MeJA. A detailed description of point-count surveys, bird species occurring along the gradient, and their feeding specialization can be found in Marki et al. (2016), Sam & Koane (2014) and Sam et al. (2017). In the 3-d survey conducted in October 2015, the total abundance of insectivorous understorey birds was recorded in 12.56 ha at each site and this figure was used in the analyses.

Due to logistical issues and time restrictions, we were not able to re-survey the ants on the focal saplings used in the current experiment, and the ant data we used here originate from the September–October 2013 survey. This approach is valid, as there were no seasonal differences observed in ant communities (Colwell et al. 2016). At each study site, we used a combination of two methods to survey ants. Tuna baits, observation and hand collection were used to detect both ant species that are, and are not, attracted to bait (Vle et al. 2009). These methods are described in detail in Sam et al. (2015a). The survey was conducted on 30 selected understorey saplings along the same transects, where the focal species *Ficus hahliana* was studied.

Statistical analyses

The data were averaged across the four experimental days because the daily number of attacks was low and did not differ significantly between the days (ANOVA; $F_{3,767} = 1.8$, $P = 0.176$). Prior to analyses, we excluded all 85 lost (missing) caterpillars (i.e. 2.21%) from the datasets, as we were not able to identify the potential predator. The effect of the study site ($N = 4$) and of the treatment (MeJA-treated vs. control) on the incidence of attacks was tested by ANOVA (function `aov` in R 3.2.4.) with nested design and two within-category effects. All the 24 sampling trees were nested within each of the four study sites. The mean daily proportions of caterpillars attacked, on each experimental tree, were arcsine transformed to meet the conditions of normality. The method of treatment was used as the first within-sampling effect and the type of predator (arthropod, bird) was used as the second. Tukey post hoc tests (package `multcomp`, function `glht` in R; Hothorn et al. 2008) were performed to inspect the differences between the study sites and the type of predator.

Results

In total, we exposed 960 artificial caterpillars at four study sites within the same time period. This resulted in 3840 caterpillar-days of exposure. In total, 1040 dummy caterpillars were attacked by a natural enemy and 2715 were not attacked during the 24-h experiments. Overall, mean \pm error daily predation was $27.6 \pm 0.98\%$ across all the trees and study sites. Across the whole gradient, the proportion of attack attempts on caterpillars exposed on the MeJA-treated trees was significantly higher (effect of treatment, Table 1; $38.2 \pm 1.48\%$ d⁻¹) than on those on the control trees ($17.4 \pm 1.31\%$ d⁻¹).

At all the study sites, the proportion of attack attempts on the dummy caterpillars exposed on MeJA-treated trees was significantly different from the proportion of attack attempts on the dummy caterpillars which were exposed on the control trees (Figure 1). The percentage of attacked caterpillars which were exposed on the control trees was significantly higher at the three lower forest study sites (i.e. 200–1700masl, 20.21–17.3% d⁻¹) than it was on the control trees at 2700 m asl (4.7% d⁻¹; Figure 1). On MeJA-treated trees, the percentage of attacked caterpillars was significantly higher at the two lower study sites (46.2–52.3% d⁻¹) than for MeJA-treated trees at 1700 and 2700 m asl (25.6–20.8% d⁻¹; Figure 1).

Most attacks on the artificial caterpillars were by arthropods (58.8% of all recorded attacks) and a lower number of attacks were made by birds (40.8%). We were not able to identify three attack marks and one other mark was made by a small mammal. On both the MeJA-treated and control trees arthropods were responsible for significantly more attacks at the lower two study sites than at the upper two sites (Figure 2).

Predation by birds was highest on the caterpillars exposed on both types of trees at 1700 m asl (Figure 2). On the MeJA-treated trees only, predation by birds reached similar levels at 2700 m asl as at 1700 m asl. The percentage of attacked caterpillars was higher at each study site in both predator groups on the MeJA-treated trees (Table 1). Significantly more attacks on caterpillars, exposed on both types of trees, were caused by arthropods than by birds in the lowlands at 200 m and 700 m asl (Figure 2). Birds were more dominant predators of caterpillars exposed on both types of trees at 1700 m and at 2700 m asl (Figure 2).

The number of caterpillars attacked by ants was highest where ants were the most abundant (i.e. lowest altitudes, Appendix 1), but the decrease in number of predation attacks by ants did not correspond significantly with the decrease in their abundance ($R^2 = 0.81$, $P = 0.10$, $N = 4$; Figure 3). Similarly, the predation rate of birds was not dependent on the number of insectivorous birds ($R^2 = 0.67$, $P = 0.18$; $N = 4$, Figure 3, Appendix 1). Due to the small number of study sites, we were not able to investigate the exact relationship statistically.

Table 1. Effect of treatment (MeJA-treated, control), altitude (200, 700, 1700, 2700 m asl) and their combined effect on overall predation on plasticine caterpillars exposed on *Ficus hahliana* along the altitudinal gradient of Mt Wilhelm by all predators together, and then separately by birds and by arthropods. Results of ANOVA with nested design (saplings by site) and two within-category (treatment and type of predator) effects.

	Overall predation				Predation by birds			Predation by arthropods		
	df	Mean Sq	F	P	Mean Sq	F	P	Mean Sq	F	P
Treatment	1	18.0	161.5	<0.001	4.9	59.9	<0.001	5.4	72.1	<0.001
Site	3	4.5	40.1	<0.001	0.7	8.9	<0.001	7.7	101.6	<0.001
Treatment × Site	3	0.9	8.8	<0.001	0.5	5.7	0.006	1.6	21.9	<0.001

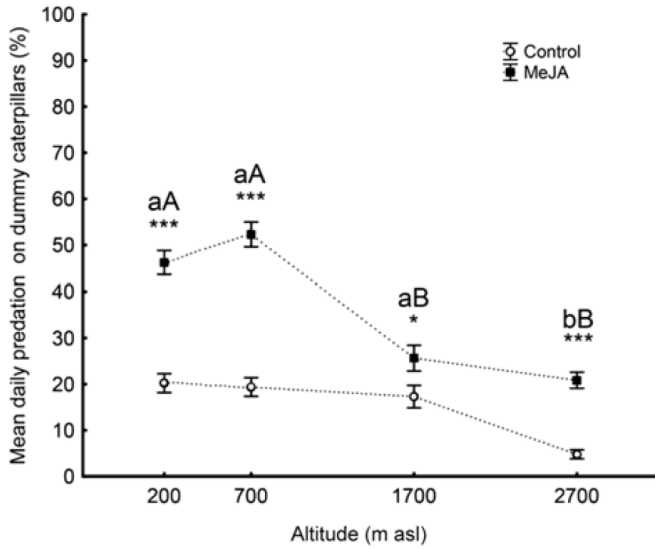


Figure 1. Mean daily (N = 4) percentage (\pm SE) of attacks by all predators on artificial caterpillars exposed (N = 5) on all control (N = 24) and MeJA-treated (MeJA) (N = 24) saplings of *Ficus hahliana* at four study sites on Mt Wilhelm in Papua New Guinea. Sites with significantly different rates of attacks

between the control and the MeJA-treated trees are marked by asterisks (***) P = 0.001, * P = 0.05; results of Tukey post hoc test). The altitudes with significantly different incidences of attack (P < 0.05) within the treatment are denoted by different letters: small letters = control saplings, capital letters = MeJA-treated saplings (results of Tukey post hoc test).

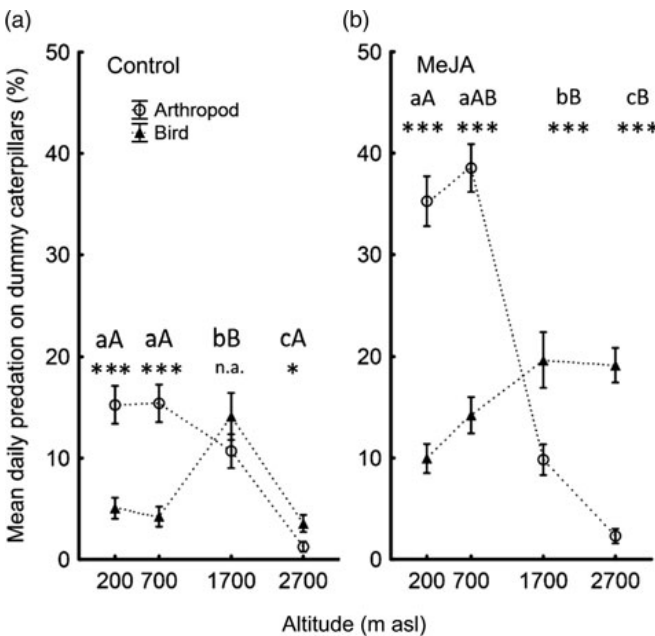


Figure 2. Mean daily (N = 4) predation by the two groups of predators on artificial caterpillars exposed (N = 5) on individual control (N = 24) (a) and MeJA-treated (MeJA) (N = 24) (b) saplings of *Ficus hahliana* at four study sites on Mt Wilhelm in Papua New Guinea. Sites with significantly different attack rates

by arthropods and birds are marked by asterisks (***) P = 0.001, * P = 0.05;

results of Tukey post hoc test). Altitudes with significantly different ($P < 0.05$) incidences of attack by individual predators within the control and MeJA treatment are denoted by different letters: small letters = predation by arthropods, capital letters = predation by birds (results of Tukey post-hoc test).

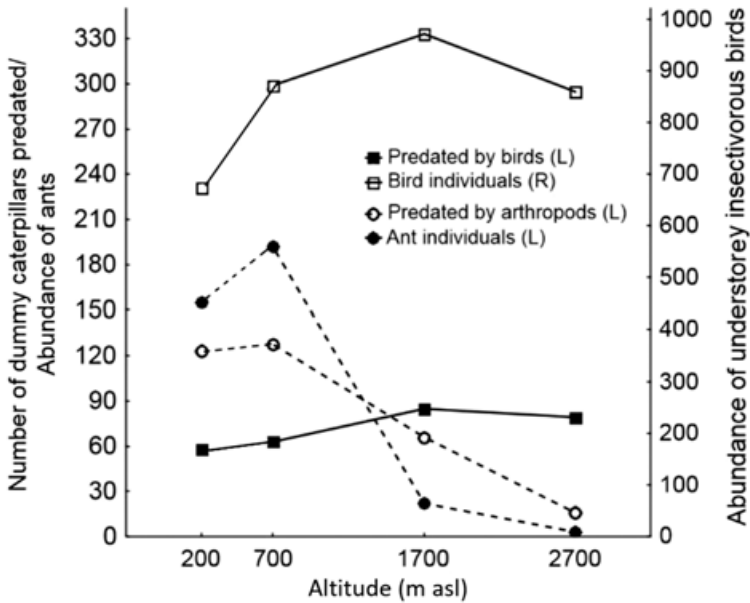


Figure 3. The total number of artificial caterpillars attacked by birds and ants (left y-axis) and the total abundance of ants recorded on the trunks of 30 understory *Ficus hahliana* trees (left y-axis) and the total abundance of understory insectivorous birds recorded in 12.56 ha during the 3-d survey (right y-axis) in Papua New Guinea.

Discussion

Altogether both groups of natural enemies inflicted about twice as much damage to the artificial plasticine caterpillars which had been placed on the MeJA-treated saplings than on the caterpillars placed on the control saplings. The effect of jasmonic treatment was important along the entire altitudinal gradient studied here, but it was relatively more important at the higher than in the lower altitudes. This would imply that plants from lower altitudes are more easily inducible than plants in higher altitudes thanks to

higher productivity at site and thus relatively lower costs of such investment into defences.

Results of observed predation levels on the MeJA-treated trees correspond with the results of other studies which report on the effect of herbivory on the predation or attractiveness of predators to herbivore-infested plants (Kessler & Baldwin 2001, Sam et al. 2015a). It is important to note that experiments using artificial caterpillars do not provide an estimate of natural predation rates, but only a relative number of predation incidents for comparison among habitats and treatments (Howe et al. 2009).

The phenomenon of induced attraction of predatory arthropods by plants in response to (simulated) herbivory is now well accepted (Agrawal 1998, Mithöfer et al. 2005, Romero & Izzo 2004, Takabayashi & Dicke 1996, Turlings et al. 1990). In previous studies, with jasmonic acid triggering a herbivore-like induced response, predatory mites were attracted by jasmonate treatment (1 mM) to *Phaseolus lunatus* in a laboratory (Dicke et al. 1999). On the other hand, predatory bugs and hoverflies were not attracted to jasmonate-treated (1 mM) *Vaccinium macrocarpon* in a field experiment. Nine out of 11 studies on parasitoid vs. jasmonate-treated plant interactions reported increased attractivity of a treated plant to parasitoids (Rodriguez-Saona et al. 2013). Our current study shows that predatory arthropods are attracted by the jasmonate treatment on *Ficus hahliana* even in complex lowland (200–700 m asl) tropical forest. The abundances of predatory arthropods (i.e. ants) at higher altitudes (above 1200 m asl) were too low to detect the effect of the treatment.

Our data also indicate that birds were able to distinguish between the MeJA-treated and intact (control) *Ficus hahliana* when they could not

see the actual herbivorous damage on the leaves. Our main result is therefore in contrast to the study conducted by Mäntylä et al. (2014). In their study, mountain birches treated with MeJA emitted more myrcene and limonene than shrubs with ongoing herbivorous damage, caused by caterpillars of the autumnal moth (*Epirrita autumnata*), and on the control shrubs. In fact, these two compounds seemed to deter birds in their study. By contrast, grey willows in the Czech Republic (Mrazova & Sam 2018), did not produce increased amounts of myrcene and limonene after the application of MeJA. Instead there were increased emissions of α -pinene which likely corresponded with the increase of predation by birds. Unfortunately, our data on volatile compounds for the current study were very limited due to contamination during a long transit. Only α -pinene, β -pinene and β -ocimene were detected in larger amounts on the MeJA-treated trees (N = 3) than on control trees (N = 2). Other detected compounds were limonene and also 3-carene which did not differ between the treatments. This report should be taken with caution.

We observed the highest predation by birds at 1700 m asl for both treatments (and at 2700 m asl for MeJA-treatment). This is roughly in the middle of the complete forest altitudinal gradient (0–3700 m asl). In previous studies, high predation by birds was found at 1700 m (Tvardikova & Novotny 2012) and between 700 and 1700 m asl of the studied gradient (Samet al. 2015a). In both studies, the predation rate by birds corresponded with the abundance of insectivorous birds, unlike the results of the current study. The authors of other studies did not find any effect of altitude on bird predation. Roslin et al. (2017) found only a weak effect of altitude on the predation by birds in a global study spanning 0–2100 m asl.

An overall decrease in predation rate with increasing altitude leads to the prediction of a higher incidence of anti-predatory defences, such as chemical or behavioural, in the lowlands, particularly against arthropods (Schmidt 1990). In a previous study from the same latitudinal gradient, a daily predation rate on caterpillars exposed on control trees at 200 m asl was $14.3 \pm 5.4\% \text{ d}^{-1}$ (Sam et al. 2015a). In the current study, predation at the same study site was $20.2 \pm 2.04\% \text{ d}^{-1}$. The predation on the control trees decreased to $2.7 \pm 0.7\% \text{ d}^{-1}$ at 2700 m asl in a previous study (Sam et al. 2015a) and to $4.7 \pm 0.97\% \text{ d}^{-1}$ in the current study at the same study site. These results suggest that the MeJA treatment tested in the current study might be slightly more attractive for predators than the simple mechanical damage used in the previous study (Sam et al. 2015a). Note that while these numbers do not represent absolute predation pressure, they are roughly comparable because they employ the same methods (plasticine caterpillars) and were conducted at the same study site (albeit in different years).

We used treatment by jasmonic acid to simulate herbivory on foliage and this may be a less efficient cue to predators than the real damage done by insectivorous herbivores (Mäntylä et al. 2014, Rodriguez-Saona et al. 2013), therefore underestimating the significance of the predator effect observed in this study. Chemically triggered responses by plants can also be expected to be weaker than mechanically simulated herbivorous damage (Rodriguez-Saona et al. 2013). To our surprise, the response of both types of predator to mechanical (Sam et al. 2015a) and MeJA-induced (this study) VOCs along the same gradient was very similar, with similar patterns for predator types in terms of qualitative observation. In both studies, the total predation on the treated trees was about twice as high.

They both also showed a decreasing trend with increasing altitude and that arthropods were relatively more dominant predators in lowlands while birds represented more important predators at altitudes above 1700 m asl.

From the literature, it is still unclear how long a tree with MeJA treatment would continue to be attractive to predators. Dicke et al. (1999) reported that there was a higher attractivity of MeJA-treated Lima beans to predatory mites for the period of 2–4 d after its application, with the strongest attraction being seen on the second day of the experiment. Mäntylä et al. (2014) applied MeJA prior to the experiment and then on days 2, 7, 9 and 11 of the experiment and observed a significant increase of predation rate between days 1 and 10 of the experiment. Rodriguez-Saona et al. (2001) treated cotton plants with MeJA overnight and observed a high production of volatiles during the daytime (7h00–15h00) on only the first day. On the grey willows growing in temperate regions an increased production of volatile compounds was detected between 30 min and 48 h after the first application of MeJA (Mrazova & Sam 2018). In our own preliminary experiments on *Ficus phaeosyce* in Papua New Guinea, the predation rate decreased significantly within the first 72 h following the first application (Appendix 2). Therefore, in the current experiment, we decided on daily re-application of MeJA.

In conclusion, our study demonstrates (1) the potential of jasmonates as a natural plant protectant against herbivorous insects via indirect defence (particularly true for arthropods but observed also in birds), (2) a decreasing attack rate of predators with increasing altitudes on the understorey *Ficus hahliana* in tropical forest, and (3) a transition in predator dominance from arthropods in the lowland forests to birds at the higher altitudes. This study shows that jasmonates provide protection

against herbivores and increase natural enemy attraction in various tropical-forest ecosystems. However, the cost of jasmonate-induced responses in the absence of herbivores remains to be studied (Baldwin 1998, Cipollini et al. 2003, Thaler 1999), and we await confirmation of whether they could lead to increased/decreased ecological costs due to the trade-offs between resistance to herbivores and pathogens (Felton & Korth 2000).

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Appendix 1

Total abundances and species richness of ants and insectivorous birds surveyed at each altitudinal study site on Mt Wilhelm in Papua New Guinea. Ant communities were surveyed by tuna baits (after 2 and 4 h) and by hand collection on 30 saplings along the transect where our study was conducted. The survey of the bird communities at each altitude was conducted at 16 points (radius 50 m, 15 min survey) regularly spaced along the 2250-m transect (i.e. on 0.1256 km²) and replicated three times. Bird species name follow IOC World Bird List version 9.1 (Gill & Donsker 2019).

Chapter III

	200 m asl	700 m asl	1700 m asl	2700 m asl
Ant diversity and abundance				
No. of individuals 2 h on tuna bait	1648	745	18	5
No. of individuals 4 h on tuna bait	1000	754	100	7
Trees with ants present – hand collection	21	14	11	2
No. of individuals collected – hand collection	155	262	22	3
Species of recorded birds				
<i>Acanthiza cinerea</i>			1	48
<i>Acanthiza murina</i>				13
<i>Aethomyias perspicillatus</i>			101	
<i>Ailuroedus buccoides</i>	2	4		
<i>Aleadryas rufinucha</i>			3	8
<i>Arses insularis</i>	3	7	5	
<i>Caligavis subfrenata</i>			1	41
<i>Campochaera sloetii</i>	1			
<i>Carterornis chrysomela</i>	13	6		
<i>Ceyx azureus</i>	8	1		
<i>Ceyx lepidus</i>	42	39		
<i>Ceyx pusilla</i>	2			

Chapter III

	200 m asl	700 m asl	1700 m asl	2700 m asl
<i>Colluricincla megarhyncha</i>	25	24	19	
<i>Coracina boyeri</i>	2	20		
<i>Dicrurus bracteatus</i>	36	10		
<i>Epimachus fastosus</i>			1	6
<i>Epimachus meyeri</i>			2	26
<i>Eugerygone rubra</i>			2	21
<i>Eulacestoma nigropectus</i>				10
<i>Gerygone chloronota</i>	4	5		
<i>Gerygone chrysogaster</i>	10	25		
<i>Gerygone palpebrosa</i>	4			
<i>Gerygone ruficollis</i>			35	49
<i>Heteromyias albispecularis</i>			8	5
<i>Ifrita kowaldi</i>				55
<i>Leptocoma aspasia</i>	48			
<i>Loboparadisea sericea</i>			1	
<i>Lophorina superba</i>			22	
<i>Machaerirhynchus flaviventer</i>	3	6		
<i>Machaerirhynchus nigripectus</i>			26	21
<i>Melampitta lugubris</i>				7
<i>Melanocharis longicauda</i>			2	
<i>Melanocharis nigra</i>	31	82	6	
<i>Melanocharis striativentris</i>			13	2
<i>Melanocharis versteri</i>			17	40
<i>Melidectes rufocrissalis</i>			68	
<i>Melidectes torquatus</i>			28	

Chapter III

	200 m asl	700 m asl	1700 m asl	2700 m asl
<i>Melidora macrorrhina</i>	2			
<i>Melilestes megarhynchus</i>	19	32	12	
<i>Meliphaga analoga</i>	83	54	29	
<i>Meliphaga aruensis</i>	2	3		
<i>Meliphaga orientalis</i>			8	2
<i>Microeca flavovirescens</i>	14	25		
<i>Microeca papuana</i>			10	28
<i>Monachella muelleriana</i>	2			
<i>Monarcha rubiensis</i>	1			
<i>Myiagra alecto</i>	11			
<i>Oedistoma iliolophus</i>		27	1	
<i>Oreocharis arfaki</i>			26	18
<i>Origma murina</i>	1	66	8	
<i>Origma robusta</i>		6	8	48
<i>Pachycare flavogriseum</i>				
<i>Pachycephala hyperythra</i>	1	2	15	
<i>Pachycephala modesta</i>				9
<i>Pachycephala schlegelii</i>			14	79
<i>Pachycephala simplex</i>		18		
<i>Pachycephala soror</i>			11	
<i>Pachycephalopsis poliosoma</i>			6	
<i>Paradisaea minor</i>	45	48		
<i>Peltops blainvillii</i>	11	5		
<i>Peltops montanus</i>				8
<i>Peneothello bimaculata</i>		35	5	

Chapter III

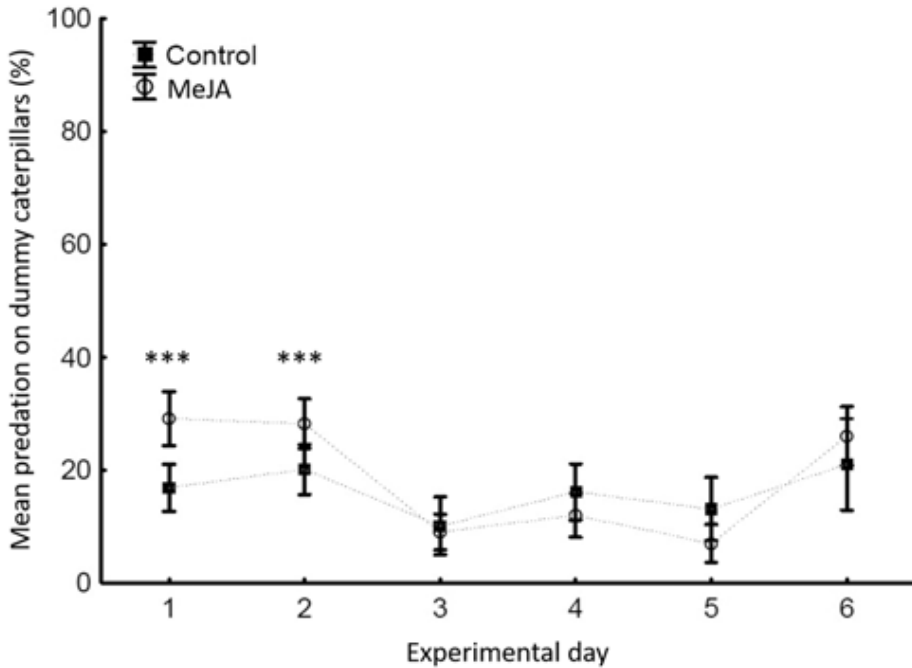
	200 m asl	700 m asl	1700 m asl	2700 m asl
<i>Peneothello cyanus</i>			94	5
<i>Peneothello sigillata</i>				66
<i>Phylloscopus maforensis</i>			10	
<i>Pitohui dichrous</i>		23	24	
<i>Pitohui ferrugineus</i>	32			
<i>Pitohui kirhocephalus</i>	12	61		
<i>Pitta erythrogaster</i>	7	12		
<i>Poecilodryas albonotata</i>				3
<i>Poecilodryas hypoleuca</i>	27	27		
<i>Ptiloprora guisei</i>			10	11
<i>Ptilorrhoa leucosticta</i>			3	6
<i>Pycnopygius ixoides</i>	4	1		
<i>Rhagologus leucostigma</i>			11	1
<i>Rhipidura albolimbata</i>			47	66
<i>Rhipidura atra</i>			54	4
<i>Rhipidura brachyrhyncha</i>			1	75
<i>Rhipidura leucothorax</i>	15	2		
<i>Rhipidura maculipectus</i>	7			
<i>Rhipidura rufidorsa</i>		6		
<i>Rhipidura rufiventris</i>	9	18		
<i>Rhipidura threnothorax</i>	19	24		
<i>Rhyticeros plicatus</i>	31	18		
<i>Sericornis nouhuysi</i>			32	49
<i>Sericornis papuensis</i>			34	20
<i>Syma megarhyncha</i>			6	1

Chapter III

	200 m asl	700 m asl	1700 m asl	2700 m asl
<i>Syma torotoro</i>	1	11		
<i>Symposiachrus axillaris</i>			9	3
<i>Symposiachrus guttula</i>	10	14		
<i>Symposiachrus manadensis</i>	29			
<i>Tanysiptera galatea</i>	10	4		
<i>Toxorhamphus novaeguineae</i>	32	59		
<i>Toxorhamphus poliopterus</i>			104	
<i>Tregellasia leucops</i>		3		
<i>Turdus poliocephalus</i>				2
<i>Xanthotis flaviventer</i>		38		
<i>Zosterops atrifrons</i>	2			
<i>Zosterops novaeguineae</i>			18	4
Abundance of birds	673	871	971	860
Species richness of birds	44	40	48	37

Appendix 2

The predation on dummy caterpillars in our preliminary experiment on *Ficus phaeosyce* was significantly higher on MeJA-treated saplings (N = 10; empty circles) than on control saplings (N = 10; filled squares) only on the first and second days (marked by asterisks: *** P < 0.001; results of Tukey post-hoc test) of the six days long experiment. MeJA was applied twice: 24 hours before the first experimental day and on the first day of study. The experiment was conducted at 150 m asl in Wanang Conservation Area in Papua New Guinea (GPS: 5°13 031.6 0 0S, 145°04 051.2 0 0E).



Chapter IV

**Specificity of induced chemical defences of two oak species
influence differently insect communities and predation.**

Anna Mrazova, Marketa Tahadlova, Veronika Rehova and Katerina Sam

**Specificity of induced chemical defences of two oak species
influence differently insect communities and predation.**

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Abstract

The multitrophic interactions are being investigated widely. Despite that, an indirect induced plant chemical defence in context of the Cry for help hypothesis is not completely understood. Insectivorous birds and arthropods use chemical cues provided by herbivory-damaged plants to find a prey. Although the specificity of herbivore-induced plant volatiles released by plants is crucial for understanding of multitrophic interactions, it remains unknown for many plant species. In our experiments, we induced English oak (*Quercus robur*) and sessile oak (*Quercus petraea*) with methyl jasmonate (MeJA) to trigger standardized chemical defensive reactions. We studied how the chemical inducible defence differs between these two closely related plant species, how it affects the predation on given species and their insect communities. Overall, in both plant species, predation was significantly higher on MeJA-treated trees than on control trees. Birds were responsible for the majority of attack attempts, followed by ants and other arthropods. The total mean amount volatiles emitted by MeJA-induced trees differed among the experimental tree species over the time and multiple applications of MeJA. MeJA application had no significant effect on abundances of insect. However, the mean body size of individuals of different feeding guilds was affected by MeJA treatment both negatively and positively, depending on specific feeding guild.

Introduction

Plants interact with other trophic levels through constitutively expressed mechanisms (Chen, 2008) which can be mechanical or chemical (e.g., leaf traits, colour, secondary metabolites) or induced upon attack (e.g., semiochemicals, herbivore-induced plant volatiles, trichomes (Mithöfer and Boland, 2012; Bandloy, et al. 2015). Chemical inducible plant defences act against herbivorous arthropods directly by synthesis of chemical compounds making the leaf tissue unpalatable or poisonous (e.g., Chen, 2008; Mithöfer and Boland, 2012; Dicke and van Loon, 2009) or indirectly by emitting volatile organic compounds trackable by various predatory taxa (e.g., Dicke et al., 2003; Heil, 2014 Mrazova and Sam, 2018, 2019; Mrazova et al., 2019).

Plant defensive chemical compounds are typically very complex, differing between plant species (Feeny, 1976; Bednarek and Osbourn, 2009; Richards et al., 2015), species growing in different habitats (Coley et al., 1985), etc. The 350 million years of continuous herbivore attacks on plants defending themselves are, in large, responsible for their gradual formation and expansion (Berenbaum and Feeny, 1981; Berenbaum and Zangerl, 1996; Benderoth et al., 2006; Bacerra et al., 2007). Ecological divergence drives species-level evolutionary diversification through the adaptability of different species' traits. Each plant species' chemical defensive compounds diversity has evolved under the selection pressure of a competitive environment (Hartmann, 1996). Moreover, the synthesis of defensive compounds is under significant genetic control (O'Reilly-Wapstra et al., 2004; Moore et al., 2014).

According to the existing knowledge, some of studied plants differ at defence strategies and intensity level within the closely related species.

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Leaves of the three tropical mangrove species were found to differ in the amount and composition of secondary compounds (McKee, 1995). Subarctic *Betula pubescens* and *Betula nana* growing in heat vegetation also differed significantly in secondary metabolites from each other (Long et al., 216). In temperate, oaks represent one of the classic model systems for elucidating the role of chemical defensive compounds in plant-herbivore interactions (Salminen et al., 24). The majority of the attention was paid to English oak (*Quercus robur*). This species was monitored closely in terms of herbivory, insect communities, and leaf chemistry (Crawley and Akhteruzzaman, 1988; Salminen et al., 24; Vehvilinen et al., 27). The leaves of English oak are soft and lack of mechanical constitutive structures avoiding herbivorous insect to feed on them. Although hydrolysable tannins, lignins, flavonoids and other chemical constitutive chemicals were recorded in the leaves (Moreira et al., 217), English oak is not known to be poisonous or repelling for the herbivorous insects. However, the variation of inducible defensive compounds of closely relative species, English oak, and Sessile oak (*Quercus petraea*) have not been emphasized yet.

Inducible chemical reactions in plants are regulated by an octadecanoid-based signalling pathway involving the wound hormone, jasmonic acid. The application of jasmonates (jasmonic acid or its volatile derivative methyl jasmonate) in low concentrations to plant leaves has been shown to induce defensive responses in plants (Boughton et al., 2003; Cheong and Choi, 2003; Hudgins et al., 2004; Belhadj et al., 2006; Parra-Lobato et al., 2009; Yu et al., 2018; Mrazova & Sam, 2018, 2019; Mrazova et al., 2019; Ho et al., 2020, Amo et al., 2022). Existing studies examining the effect of MeJA on plants showed that the reaction (i.e., amount of emitted volatile defensive compounds) of artificially and long-term

stressed plants (i.e., repeated MeJA applications) increases with time regardless of plant species (e.g., Mrazova & Sam, 2018).

The effect of MeJA on insect abundances remains unresolved. Despite a growing number of studies investigating the effect of external application of MeJA, results are still contradictory. The studies published to date report both no effect of external application of MeJA on insect performance, mortality, or abundance (e.g., Tan et al., 2011; Williams et al., 2017) and a significant repellent effect (e.g., Tan et al., 2011). If MeJA has no effect on overall insect abundance on the host plant, the reason may be the replacement of individual insect guilds. While herbivorous insects may be repelled by the application of MeJA indirectly through the effect of defence mechanisms triggered in the plant, invertebrate predators, according to the Crying for help hypothesis, may instead be attracted to the induced chemical volatile compounds. The resulting insect abundance thus remains unchanged. Although there are no studies investigating the effect of MeJA- or herbivore-treated plants on the average body size of herbivores to our knowledge, it can be assumed that the average body size of herbivores feeding on the defending plant might be smaller due to the negatively altered edibility of the foliage of the defending plant.

According to the Crying for help hypothesis, predators and parasitoids of herbivorous insect can use defensive volatile compounds released by herbivore-attacked plants as a cue to finding their prey or hosts (Baldwin & Schulz, 1983). It has been well documented that insectivorous invertebrates: predatory mites (Dicke and Sabelis, 1988), parasitoid wasps (Turlings et al., 1990), predatory bugs (Drukker et al., 1995), predatory lady beetles (Ninkovic et al., 2001), nematodes (Rasmann et al., 2005); and birds (Mäntylä et al., 2008) are attracted to herbivore-damaged plants (see review Heil et al., 2014). Oaks, considered to be one the most abundant

deciduous tree species in Europe, host diverse insect species of high abundances and are thus a keystone species for multitrophic interactions functioning (Schafale and Weakley, 1990).

Hypotheses

Based on different constitutive performance of studied oak species, we assume that English oak invests into inducible defence and responds more intensively to induction by MeJA than sessile oak. As methyl jasmonate showed to be a reliable inducer of plant defensive reactions, we hypothesise that MeJA-treated individuals of both oak species are more attractive for predators of herbivorous insects than intact trees. Moreover, defensive reaction of both studied oak species increases with time and replications of the application of MeJA if reapplied long-term. We also assume that intact and MeJA-treated English oak is more attractive for insectivorous predators than intact and MeJA-treated sessile oak respectively due to less developed constitutive defence in English oak. MeJA treatment has a positive effect on abundances of predatory arthropods which might be attracted by increased emissions of volatile defensive compounds. Abundances of non-predatory arthropods are indirectly affected by increased number of predatory arthropods in response to MeJA treatment. Thus, we believe that the total arthropod abundances are not affected by MeJA treatment due to mesopredators release effect. We also assume that the mean body size of arthropod communities is affected by MeJA treatment. It is likely that body size of chewers will decrease as the leave tissue of induced trees is less palatable, than the leaf tissue of healthy trees, while the mean body size of other feeding guilds remains unchanged.

Methods

Study site and species

The study site was located within a mixed temperate forest near České Budějovice, Czech Republic in spring 2020 (GPS: 48.966817, 14.350394). Specifically, the experiment was conducted in a large forest gap (ca. 100 x 40 m) surrounded by a structurally diverse mixed forest with tree ages ranging from ca. 10 to 70 years, which supported high diversity and abundance of birds and insects in different vertical strata.

For the experiment we used two closely related oak species which often hybridize (Muir et al., 2001): English oak (*Quercus robur*) and sessile oak (*Quercus petraea*). We purchased 60 young saplings (1.5-2m) of both species planted in 2l buckets in March 2020. We then transplanted them into 80 litre pots filled with standard soil (AGRO horticultural substrate, Czech Republic) at the beginning of March and placed them in the premises of the Biological Centre of the Academy of Sciences of the Czech Republic in České Budějovice, where we watered them regularly. We moved the experimental saplings to the study site after their leaves had fully developed (28th April 2020). The experimental trees were arranged in 10 rows of 6 with a minimum distance of 5 m to be considered independent. Half of all trees were selected as to be MeJA-sprayed, while the rest served as control. We arranged these trees regularly starting from the first row with sessile oak control, MeJA-treated sessile oak, English oak control, MeJA-treated English oak, etc., to cover the entire study area (Fig. S1). For the following two weeks, we left the trees in the plot to acclimatise. This time also allowed the natural predators to get used to the trees newly appeared in the forest gap.

The experiment was conducted from 18th to 31st of May 2020. The first four days of the experiment were sunny with an average daily temperature of 22 °C. From the 5th to the 10th day, showers

occurred with occasional persistent rain and cooling to an average daily temperature of 16 °C. From day 11 to the end of the experiment, the weather was again rain-free with a consistent daily mean temperature of 19 °C.

Experimental setup

The experiment was conducted from 18th to 31st of May 2020. The day before the start of the experiment (day 0) and every 24 h thereafter, the leaves of all MeJA- treated trees were sprayed with 30 ml of a freshly prepared 15 mM methyl-jasmonate solution (95% MeJA Sigma Aldrich 392707) consisting of 94.55% distilled water, 5% ethanol, 0.35% MeJA, and 0.1% Tween20 from Sigma Aldrich; following the previous method of Mäntylä et al., 2014). To avoid the differences in reflectance between dry and wet leaves, we sprayed the leaves of all control trees with 30 ml of distilled water following the MeJA treatment schedule.

To assess the attractiveness of MeJA-treated trees to herbivore insect predators, we used plasticine caterpillars made from modelling clay (Koh-I-Noor Hardtmuth, České Budějovice, Czech Republic) as a surrogate for herbivore insects. Following the recommendations from the previous studies using this methodology (Lövei & Ferrante, 2017; Roslin et al., 2017), the light green clay was pressed through a stainless-steel sugar paste extruder (Antoble) to obtain artificial caterpillars with a smooth surface (diameter = 3 mm, length = 20 mm). The artificial caterpillars resembled generic inconspicuous lepidopteran larvae commonly found on oaks in Central Europe (e.g., Autumn moth - *Operopthera autumnata*).

On the first day of the experiment (day 1), we randomly attached 5 artificial caterpillars at the base of leaves by an entomological pin (Sphinx

01.10 size 2; Czech Republic), so the caterpillars are at least 30 cm apart. Every 24 h, we collected artificial caterpillars to entomological box and kept for further identification of the marks according to the published keys (Howe et al., 2009; Low et al., 2014). Caterpillars with visible damage were replaced by new ones, which we placed to a slightly different location. In total, 300 plasticine caterpillars were exposed on the trees at any one time. We then determined the predator responsible for the infestation based on the type of bait damage (Howe et al., 2009; Low et al., 2014; Sam et al., 2015). In total, we obtained data on predation from 3,600 caterpillar-days throughout the experiment (i.e., 300 artificial caterpillars exposed for 12×24 hours).

Collection of plant volatiles

To describe any differences in the production of defence chemicals in the two closely related oak species over the time and between each other, we conducted three collections (days 2, 6, and 11 of the experiment) of the volatile compounds emitted by the leaves. We randomly selected 4 MeJA-treated trees and 4 control trees of each of the plant species (i.e., 16 samples in total) to sample. We collected two parallel, validation samples of ambient air, resulting thus into 20 samples taken on each sampling day. Each sampling was done before this day's MeJA application.

To collect the leaf volatiles, a branch with approximately 50 leaves was placed in a polyethylene terephthalate (PET) bag (Tesco, Poland, Kraków; 35 x 43 cm) and sealed with a plastic tightening strap. The bag was allowed to fill passively with leaf volatiles for 60 min, after which we cut off one corner of the bag and inserted a glass thermal desorption (TD) tube containing approximately 100 mg of Tenax TA adsorbent (Supelco,

mesh 60/80; Bellefonte, PA, USA). The TD tube was connected to a vacuum pump (PAS-500, Spectrex, Redwood City, CA, USA) via conditioned silicone tubing. Air was drawn through the TD tube at a flow rate of 200 ml/min. Active sampling of volatiles was conducted for 15 min and a total of 3 L of air was drawn through each tube. The TD tubes were then removed, sealed with silicone caps, and immediately sent for analysis by gas chromatography-mass spectrometry to the Institute of Environmental Technology in Ostrava-Poruba, Czech Republic.

Analysis of plant volatiles

Prior the experiment, TD tubes were conditioned with a gas chromatograph (5890 Agilent, modified for spike and cleaning TD tubes) for 30 minutes at 320 °C and a 20 ml/min Helium flow rate at the Institute of Environmental Technology in Ostrava-Poruba, Czech Republic. The VOCs adsorbed to TD tubes were analysed following the methods described in Amo et al. (2022); see Appendix S2 for details.

Insect communities

Four days after the end of the experiment (i.e., day 17), we collected insects from all trees using a standardize beating methods (3 hits by a stick to the crown of each sapling above 1.5 × 1.5 m beating sheet). We captured all arthropods fallen, and we visually search for individuals rolled in leaves or firmly attached and placed them into labelled vials filled with 70% ethanol. We also estimated the number of leaves of each of the tree as an indicator of relative insect abundance. During these four days, no more plasticine caterpillars were present on the trees and the trees were not treated in any way. Further, we identified all insects into genera and

feeding guilds (Tab. 1).

Table 1: An overview of arthropod taxa and their classification in feeding guilds.

Guild	Taxon	
Gallers	Cynipini	
Leaf chewers	Chrysomelidae	
	Curculionidae	
	Lepidoptera	
	Symphyla	
Miners	Agromyzidae	
	Cecidomyiidae	
NR	Nosodendridae	
	Buprestidae	
	Cantharidae	
	Cerambycidae	
	Cleridae	
	Cryptophagidae	
	Elateridae	
	Melandryidae	
	Mordellidae	
	Psocoptera	
	Ptinidae	
	Simuliidae	
	Parasitoids	Chalcidoidea
		Chalcidoidea -
Pteromalidae		
Dryinidae?		
	Megastigmidae?	
Predators	Araneae	
	Coccinellidae	
	Ephidridae/Ochthera	
	Formicidae	
	Syrphidae	
Sap suckers	Auchenorrhyncha	
	Heteroptera	
	Sternorrhyncha	
	Thysanoptera	

Statistical analyses

To select the best predictors for each selected dependent variable, we followed a simple procedure. First, we visually checked the raw data using *ggplot2* package. Second, we created a generalized linear model (glm) or a generalized linear mixed model (glmm) using the *lme4* (Bates et al., 2015) or *glmmTMB* package (Brooks et al., 2017). This “full model” contained variables Species (factor of two levels), Treatment (factor of two levels), Compound (factor of ten levels), Guild (factor of four levels) and all their possible interactions. As a random effect, we used Tree identity and Day (Table 2). Model fit was then analyzed using *check_model* and *check_distribution* functions from the *performance* package (Lüdecke et al., 2020), and distribution of residuals was visually examined. To reduce unnecessary predictors, we used the *dredge* function from the *MuMIn* package (Barton, 1986), which creates a model for each possible combination of presence of each predictor and selects the best model using parsimony (AICc). If multiple models result in similar AIC score (delta AIC < 2), we compared those models using function AICtab (package *bbmle*, Bolker et al. 2020). When the best model was picked, we analyzed the model fit again using *check_model* and *check_distribution* functions from the *performance* package (Lüdecke et al., 2020), and distribution of residuals was visually examined again. For the final model, we tested included effects by conditional F-tests with the Satterthwaite’s or Kenward Roger approximation, or Chi-square tests using *drop1* function (Chambers, 1992). And second, obtained estimated marginal means (= emmeans) and their post-hoc t-test for the pairwise comparisons, using the *emmeans* package (Lenth, 2020). The most parsimonious models are included in Table (Table 2). Due to the visible differences in the raw data, we did no

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follow the rule of the smallest dAICc and we tested for the estimated marginal means of a respective interaction in three models (Table 2). Throughout the whole text, results are indicated by asterisks denoting the following levels of significance *** $p < 0.001$, ** $p < 0.01$, * $p < 0.5$.

Table 2: Model selection overview. The values represent dAIC of the respective models, where zero represents the best model (see Method) and “NR” represents not relevant variable(s) for the respective analysis.

Explained variable	HIPV compounds' emission			Mean plasticine caterpillar predation			Abundance			Arthropod
	Series 1	Series 2	Series 3	Bird + Arth.	Arth.	Bird	Total	Guild comparison	Predators/all ratio	Mean body size
Model details	Gamma, log-link			Binom., logit-link			N. binom., log-link		Binom., logit-link	Normal, log-trans.
Random factor(s)	TreeID	TreeID	TreeID	TreeID + Day	TreeID + Day	TreeID + Day	NR	TreeID	NR	TreeID
Explanatory variables										
Null	179.3	114.72	121.84	24.64	14.36	11.19	65.32	103.62	0	1277.44
Compound	0	0.28	8.03	NR	NR	NR	NR	NR	NR	NR
Treatment	NR	NR	NR	2.63	0	4.04	66.18	105.1	1.27	75.22
Compound+Species	2.28	0	8.53	NR	NR	NR	NR	NR	NR	NR
Compound+Species+Compound:Species	17.55	2.13	0	NR	NR	NR	NR	NR	NR	NR
Species+Treatment	NR	NR	NR	0	1.83	0	11.13	3.44	3.56	1287.55
Guild+Species	NR	NR	NR	NR	NR	NR	NR	0	NR	26.8
Species+Treatment + Species:Treatment	NA	NA	NA	1.9	3.11	2.03	0	3.44	5.4	82.58
Species+Guild +Guild:Species	NR	NR	NR	NR	NR	NR	NR	0.15	NR	78.68
Guild+Species+Treatment+ Guild:Treatment +Guild:Species+ Treatment:Species + Guild:Species:Treatment	NR	NR	NR	NR	NR	NR	NR	13.14	NR	0

Results

Predation

We exposed a total of 300 artificial caterpillars daily. Over the 12 days of the experiment, this accumulated into 3600 caterpillar exposition days. Overall, we detected 136 (3.85%) caterpillars with visible predation marks, for which were responsible predominantly birds (78; 2.21%) and ants (38; 1.08%). Wasps and other unspecified arthropods attacked 20 (0.57%) caterpillars. The 65 (1.81%) caterpillars that were completely missing or found on the ground were excluded from the analysis of predation results.

The overall predation did not differ among species significantly. However, artificial caterpillars exposed on English oak had 1.7 times higher chance to be attacked than caterpillars exposed on sessile oak. The chance of attack increased three times after MeJA application (Tab. 3; Fig. 1).

Table 3. Effect of Treatment (MeJA, control), Species (*Q. Robur*, *Q. Petraea*) and their combined effect on overall predation on plasticine caterpillars exposed on two oak species by all predators together, and separately by birds and arthropods. Results of ANOVA (*binomial glmer model with the species and treatment as fixed factors, and tree individual and day as random factors*).

	Overall predation			Predation by birds			Predation by arthropods		
	χ^2	<u>Df</u>	p	χ^2	<u>Df</u>	p	χ^2	<u>Df</u>	p
Treatment	24.6	1	< 0.001	9.2	1	< 0.01	15.9	1	< 0.001
Species	4.6	1	< 0.05	5.8	1	< 0.05	0.2	1	0.7
Treatment : Species	0.1	1	0.7	–	–	–	–	–	–

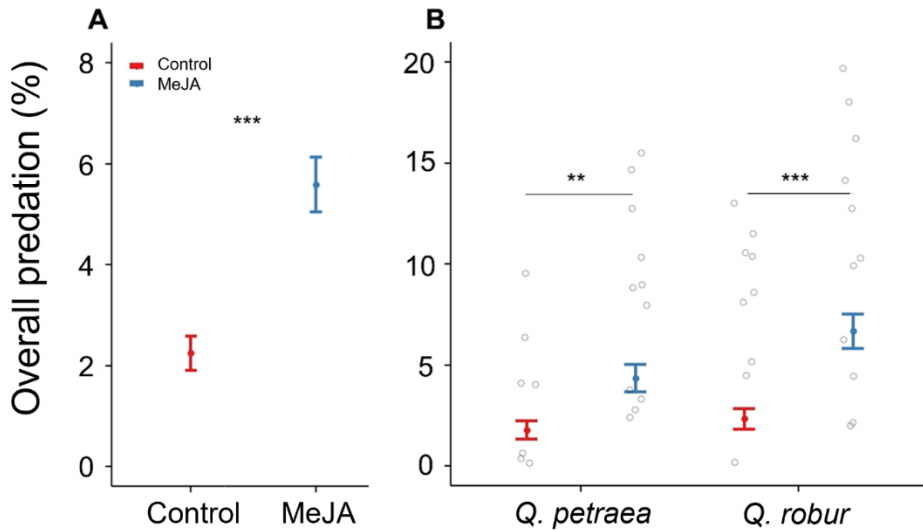


Figure 1: The overall mean (\pm s.e.) daily percentage of attacked caterpillars exposed on the two oak species was higher on trees treated with methyl jasmonate (**A**: $p < 0.001$; **B**: *Q. petraea*: $p < 0.01$; *Q. robur*: $p < 0.001$).

Birds were responsible for most of the attacks on attacked artificial caterpillars (57.4%; Tab. 3, Fig. 2A). The percentage of artificial caterpillars attacked by birds was significantly higher on MeJA-treated trees ($p < 0.05$) only in the case of English oaks but not on sessile oaks (Fig. 2B). Application of MeJA had a significant effect on the increase in the percentage of caterpillars attacked by arthropods (Tab. 3) in both Sessile oak ($p < 0.05$) and English oak ($p < 0.001$) (Fig. 3B). Specifically, ants were responsible for 27.9%, unspecified arthropods and parasitoids for 14.7% of all attacks respectively (Fig. 3B).

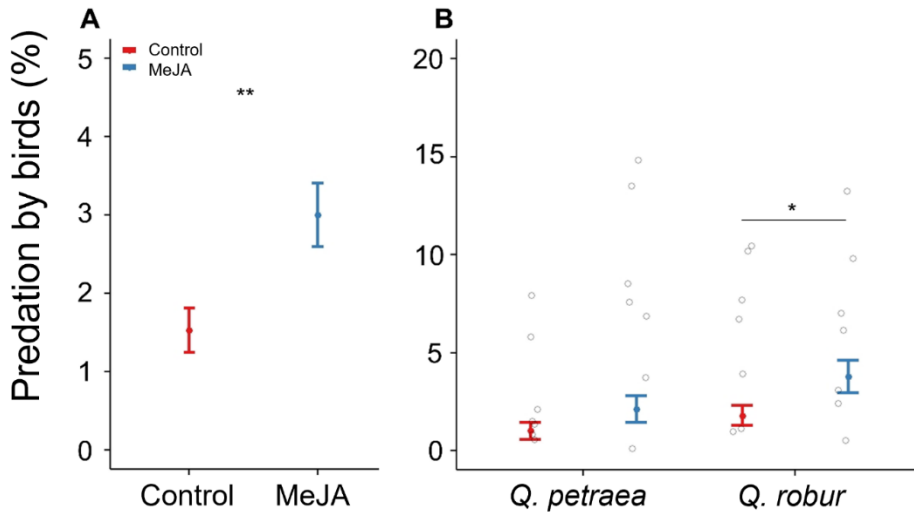


Figure 2: The mean (\pm s.e.) percentage of caterpillars attacked by birds exposed on the two oak species was higher on trees treated with methyl jasmonate (**A**: $p < 0.01$). Birds predated significantly more caterpillars exposed on MeJA-treated than on control *Q. robur* (**B**: $p < 0.05$), whereas predation on *Q. petraea* did not increase significantly after MeJA application.

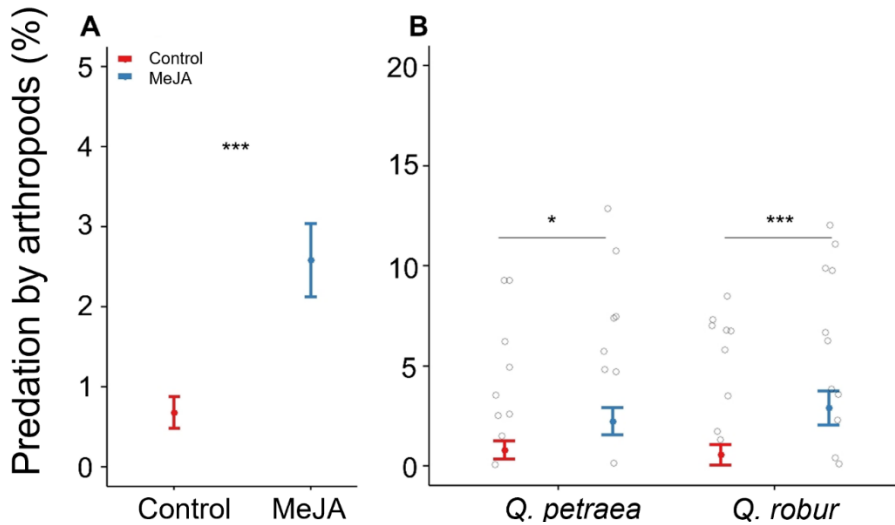


Figure 3: The mean (\pm s.e.) percentage of arthropod-attacked caterpillars exposed on the two oak species was higher on trees treated with methyl jasmonate (A: $p < 0.001$; B: *Q. petraea*: $p < 0.05$; *Q. robur*: $p < 0.001$).

Analyses of volatile compounds

Analyses of VOCs detected tens of different compounds. We focused on terpenes, which are associated with chemical induction of plants. We compared the concentrations of four selected terpenoids (α -pinene, β -pinene, 3-carene, and D-limonene) within samples collected from control trees. Volatiles production did not differ in quantity or quality between control tree species (ANOVA $\chi^2 = 0.0016$, Df = 1, $p = 0.97$). After the MeJA application, MeJA-treated trees newly synthesized 10 compounds

that was not recorded for control trees and differed quantitatively between the species studied and over time and multiple MeJA applications.

While the total mean amount of emissions produced by MeJA-treated sessile oak increased over time, the amount of compounds emitted by English oak decreased (Fig. 4). The total mean amount of MeJA-induced volatiles differed among the experimental tree species at Series 1 (24 h after the first and the only application; Tab. 4; Fig. 5) Series 2 (96 h after the first application and thus after four applications; Tab. 4; Fig. 6) and series 3 (216 h after the first application and thus after 9 applications; Tab. 4; Fig. 7) measurements.

Table 4: Effect of Compound, Species (*Q. Robur*, *Q. Petraea*) and their combined effect on total emissions sampled three times over the course of 13 days long experiment. Results of ANOVA (linear mixed model to predict \log_2 (Amount) with Compound and Species (formula: Amount ~ Compound + Species + Compound:Species). The model included treeID as random effect (formula: ~1 | treeID).

	Series 1			Series 2			Series 3		
	F	Df	p	F	Df	p	F	Df	p
Compound	75.5	9	< 0.001	40.3	9	< 0.001	41.1	9	< 0.001
Species	0.3	1	0.6	1.5	1	0.3	2.1	1	0.2
Compound : Species	1.1	9	0.4	2.3	9	< 0.05	3.2	9	< 0.01

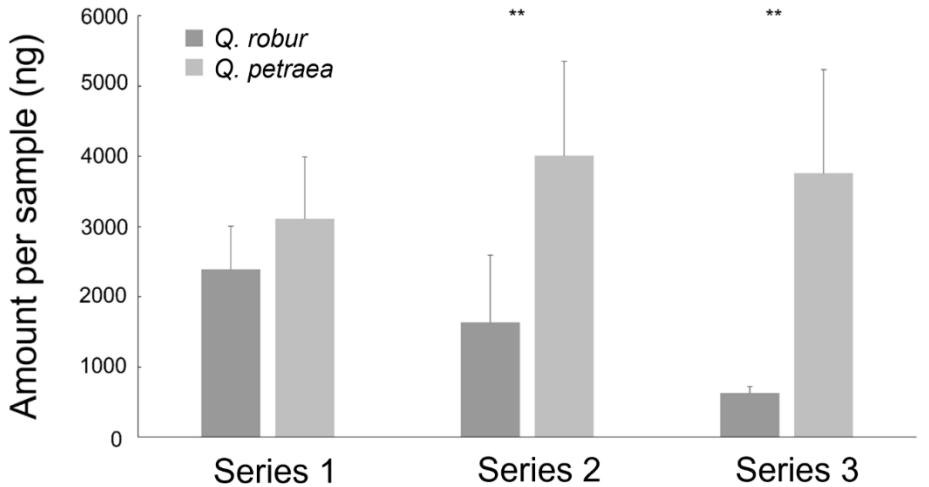


Figure 4: Comparison of the total average amount (\pm s.e.) of emissions of individual samplings for English oak (*Q. Robur*) and sessile oak (*Q. petraea*). The total amount of emitted compounds decreased over time for *Q. Robur* and increased for *Q. petraea*. Between plant species, the differences in total amounts of emitted compounds were significant during the Series 2 and Series 3 ($P < 0.01$). Series 1 = 24 hours after the first spraying, series 2 = 96 hours after spraying and 4 MeJA applications, series 3 = 216 hours after spraying and 9 MeJA applications.

beta-ocimene, trans beta-ocimene and linalool, increased emissions of caryophyllene and humulene were observed. The third sampling after 216 h showed also significantly higher emissions of beta-phellandrene in English oak and beta-ocimene, trans beta-ocimene, gemarcene and beta-bourbonene in sessile oak (Tab. 4, Fig. 7). The amount of sampled HIPVs is not correlated with the number of leaves growing on both oak species. Tested by the method of Pearson's correlation ($R = -0.24$, $p = 0.21$; Fig. S3).

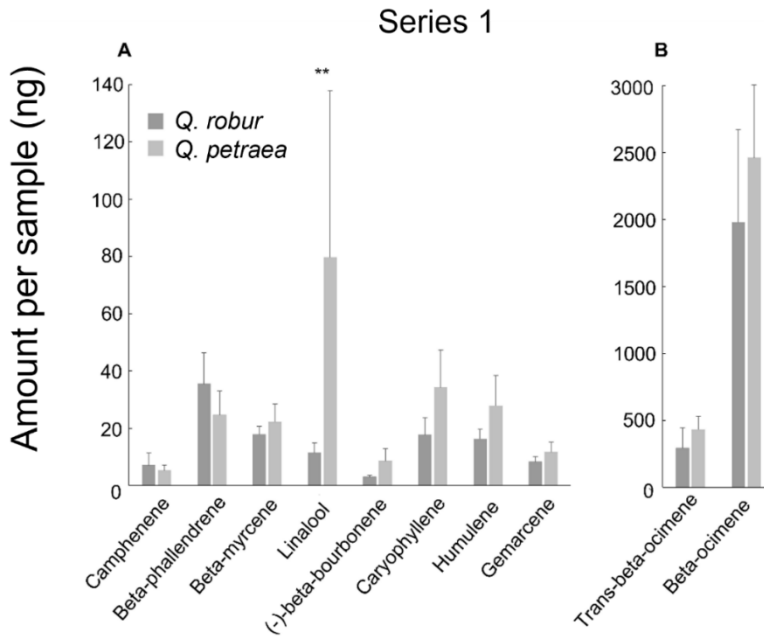


Figure 5: Comparison of specific defensive compounds emitted by MeJA-treated English oak (*Q. robur*) and sessile oak (*Q. petraea*) 24 hours after the first and only MeJA application. Sessile oak emitted significantly higher amount of Linalool.

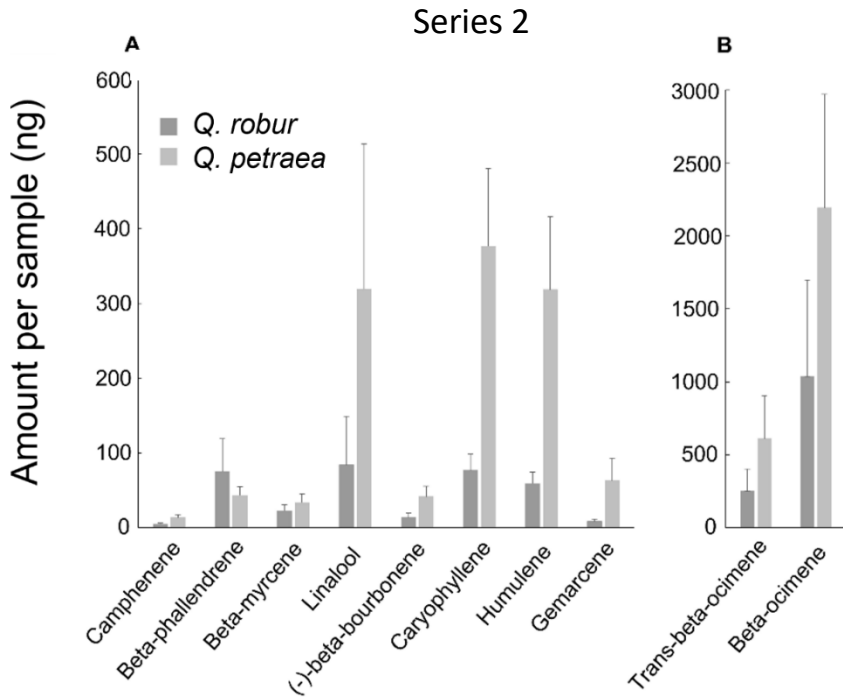


Figure 6: Comparison of specific defensive compounds emitted by MeJA-treated English oak (*Q. robur*) and sessile oak (*Q. petraea*) 96 hours after the first and 4 MeJA applications in total. The amounts of studied compounds did not differ between oak species.

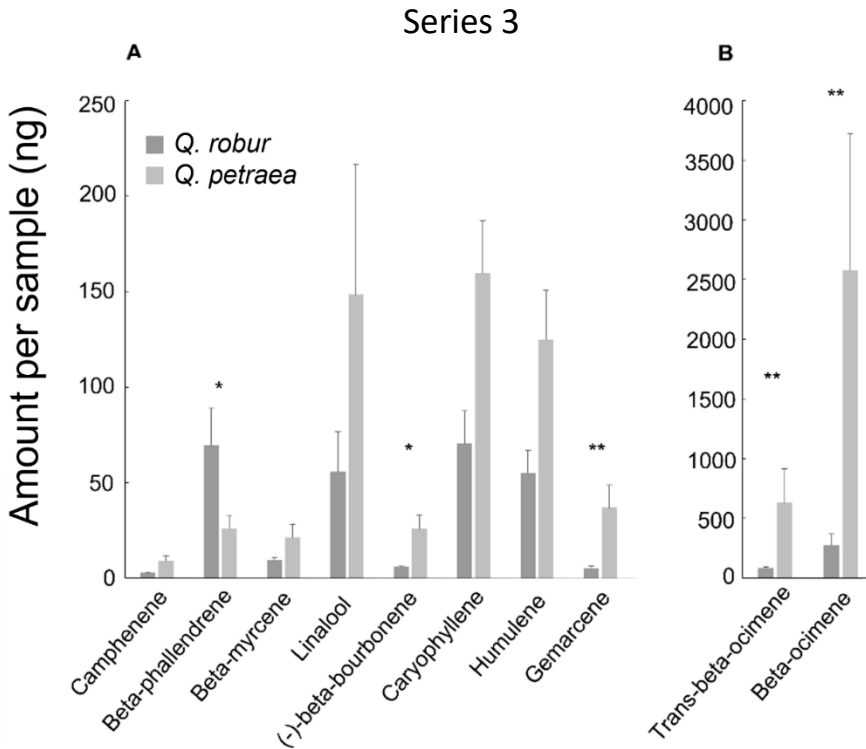


Figure 7: Comparison of specific defensive compounds emitted by MeJA-treated English oak (*Q. robur*) and sessile oak (*Q. petraea*) 216 hours after the first and 9 MeJA applications in total. Sessile oak emitted significantly higher amounts of (-)-beta-bourbonene, Germacrene, Trans-beta ocimene, and Beta-ocimene in contrast to English oak, while English oak emitted higher amounts of Beta-phellandrene.

Insect communities

We collected 1300 arthropod individuals in total. Abundance of arthropods marginally significantly increased on English oaks than on sessile oaks regardless of MeJA induction (ANOVA $\chi^2 = 3.4413$, Df = 1, $p = 0.06$; Fig.

8). Abundance of arthropods did significantly differ between the MeJA-treated oak species (ANOVA $\chi^2 = 4.14$, Df = 1, $p < 0.05$). Specifically, abundance of predatory arthropods (including ants and spiders) differed significantly between the oak species (Contrasts of marginal effects ratio = 0.615; $p = 0.02$; Fig. 9).

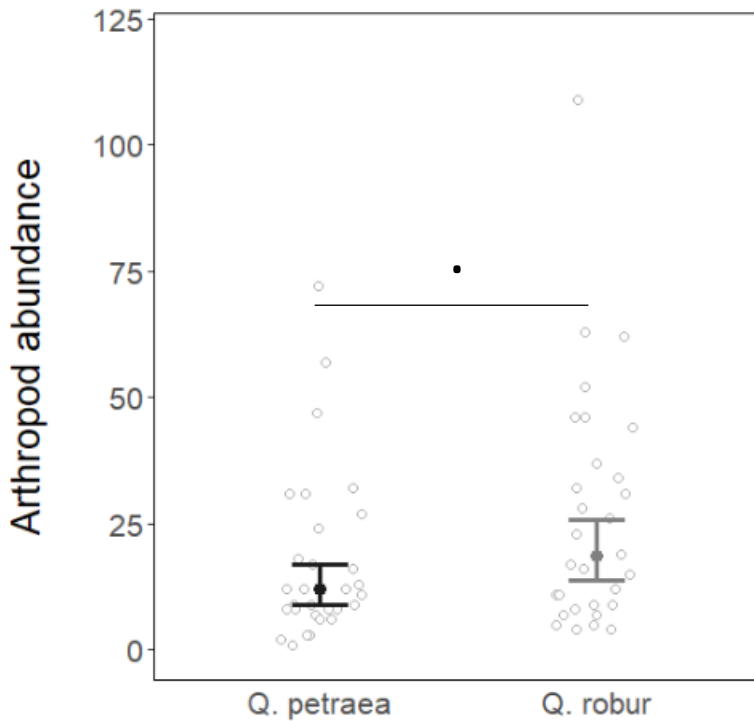


Figure 8: The mean (\pm s.e.) abundance of arthropods sampled per tree was higher on English oak (*Q. robur*) in comparison to sessile oak (*Q. petraea*). The result is indicated by a dot denoting the level of marginal significance of $p = 0.06$.

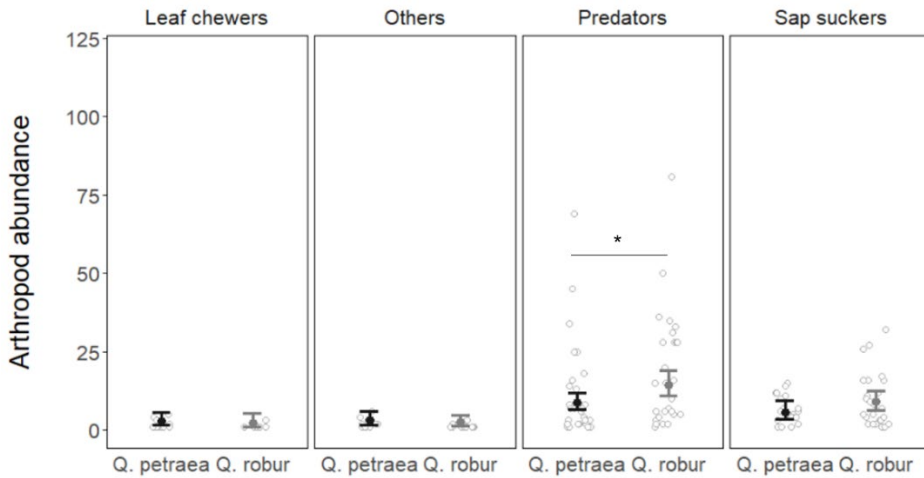


Figure 9: The mean (\pm s.e.) abundance of arthropod predators differed significantly between the oaks.

MeJA treatment had no effect on mean abundance of arthropods (ANOVA $\chi^2 = 0.96$, Df = 1, $p = 0.33$). However, MeJA treatment had a significant effect on mean body size of individual guilds (ANOVA $F = 15.472$, Df = 3, $p < 0.001$; Fig. 10). Specifically, for both oak species, the mean body size decreased significantly in chewers after MeJA application. The mean body size of sap suckers and other arthropods in English oak and predatory and other arthropods in sessile oak has increased. Application of MeJA had no effect on mean body size of predatory arthropods for English oak, and of sap suckers for sessile oak (Tab. 5; Fig 10).

Table 5: The effect of MeJA application on mean body size of arthropods and its comparison between English oak (*Q. robur*) and sessile oak (*Q. petraea*). Contrasts of log (body size), with Treatment, Guild and Species and their combine effects as fixed factors and arthropod individual as random factor (formula: $\log(\text{Size}) \sim \text{Treatment} + \text{Guild} + \text{Species} + \text{Treatment:Guild} + \text{Species:Guild} + \text{Species:Treatment} + \text{Treatment:Species:Guild} + (I | \text{Individual})$).

Treatment	Species	Guild	df	t.ratio	p.value
Control / MeJA	<i>Q. petraea</i>	Chewers	485.5829	3.260544	0.00119
	<i>Q. robur</i>		1137.294	2.221026	0.026546
	<i>Q. petraea</i>	Sap suckers	101.2342	-0.33383	0.7392
	<i>Q. robur</i>		69.76127	-2.92794	0.004605
	<i>Q. petraea</i>	Others	435.9556	-1.96216	0.050379
	<i>Q. robur</i>		754.5157	-7.08314	3.23E ⁻¹²
<i>Q. petraea</i>	Predators	71.20722	-2.19178	0.031667	
<i>Q. robur</i>		58.63503	0.454816	0.650921	

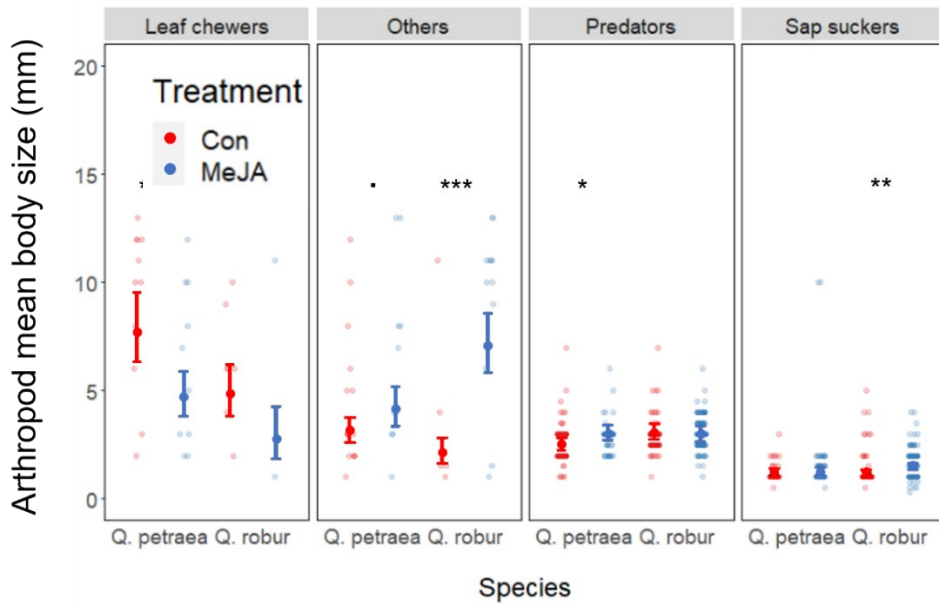


Figure 10: Comparison of MeJA application effect on mean body size of arthropods sampled from English oak (*Q. robur*) and sessile oak (*Q. petraea*). For both oak species, the mean body size decreased significantly in chewers. The mean body size of sap suckers and other arthropods in English oak and predatory and other arthropods in sessile oak has increased. Application of MeJA had no effect on mean body size of predatory arthropods for English oak, and of sap suckers for sessile oak. Results are indicated by asterisks and a dot denoting the following levels of significance *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, • $p = 0.0504$.

Discussion

Our experiment concludes that application of methyl jasmonate leads to increased attractiveness of trees to insectivorous predators including invertebrates and birds. This supports the results of Mrazova & Sam (2017) on willows, and Volf et al. (2021) on English oak but contradicts studies by Saavedra & Amo (2018) or Mäntylä et al. (2014). In contrast to our experiment, Saavedra & Amo (2018) conducted their experiment in spring in a study plot in an oak forest (Sierra de Guadarrama, central Spain, 40° 43'N, 03° 55'W), within a period when a lot of other food in the vicinity was available. In this study, only predation by insectivorous birds was considered, while attacks by predatory arthropods were disregarded. Given that arthropod predation typically represented about 30% of all attacks in our previous studies (Mrazova & Sam, 2018, 2019.), we can assume that results of Saavedra & Amo (2018) were significantly affected. A likely for inconsistency of Mäntylä's et al. (2014) study with our results may be that they only induced by MeJA one experimental branch with a larger tree, whereas we treated the whole tree. Recent results of Volf et al. (2021) suggest that there is a significant level of localization in induction of VOCs in oak trees and probably also in unknown traits with direct effects on herbivores. This study tested the defensive response of individual branches of mature English oak treated with MeJA, thus, we propose to focus future experiments on the differential effects between topical application of MeJA to branches and to whole trees.

Surprisingly, the overall predation rate on artificial caterpillars did not significantly differ between oak species. Given the high abundance of insects on the host plant and its high inducibility, we expected a higher

predation rate of caterpillars exposed on English oak in comparison to sessile oak. Over the duration of the experiment, we observed a decrease in predation rate in the middle of the experiment (ca. after 6 days). Over the course of the experiment, predators might learn that exposed caterpillars are artificial and began to avoid it. Subsequently, however, the predation rate began to rise again to the level of the first days of the experiment in case of both oak species and treatments (Fig. S4). Thus, the reduced predation rate in the middle of the trial was probably due to the weather, which was rainy and cold at the time. Mrazova & Sam (2017) compared their results to the weather and found that morning rain reduced morning activity of birds and arthropods.

The analysis of the volatile compound showed that the naturally emitted blend of VOCs from control trees are not significantly different between studied species. This result is little bit surprising as English oak emitted more terpenes than sessile oak in the Mediterranean region (Csiky & Seufert, 1999; Steinbrecher et al., 1997). However, source of this discrepancy might be given by the latitudinal difference between the sites. The theory of latitudinal gradients in plant defence proposes that plants at low latitudes develop stronger defences as adaptation to higher levels of herbivory by developing. Alternatively, another explanation might be the uniformity of our soil. While English oak grows in sandy soils and loamy sand substrates, sessile oak prefers soils with higher pH (Pietrzykowski et al., 2015; Röhrig et al., 2020).

A significant interspecific difference in the amount of emitted defensive chemicals is shown after application of methyl jasmonate. Both oak species released a spectrum of compounds that the control trees did not. These compounds were qualitatively the same for both oak species but

differed in the amounts. The sessile oak produced higher quantities of VOCs which were increasing over the duration of the experiment, while the amount of VOCs emitted by English oak was lower and decreased over the duration of experiment. Interestingly, although the sessile oak showed higher VOC emissions after methyl jasmonate application, a higher predation rate of invertebrate predators as well as birds was observed on the English oak. This implies that higher emission rates do not always mean greater attractiveness to predators.

MeJA-treated trees emitted 10 specific compounds that were not detected in control trees. Specifically, amounts of the beta-ocimene and trans beta-ocimene increased the most significantly on MeJA-treated trees in contrast to controls. Linalool, caryophyllene and humulene were also significantly abundant, but emitted in lower amounts than beta-ocimene and trans-beta ocimene. Similarly, Mountain birch (*Betula pubescens*) infested by live caterpillars of autumnal moths (*Epirrita autumnata*), produced significantly higher amounts of beta-ocimene, linalool and beta-caryophyllene than uninfested trees (Mäntylä et al., 2008). Beta-ocimene was also found to be induced after MeJA-application to grey willow (*Salix cinerea*), despite mainly α -pinene, β -pinene, 3-carene and limonene emissions increased in this experiment (Mrazova & Sam, 2017). Comparison of the reaction of Pyrenean oak (*Quercus Pyrenaica*) to MeJA and live herbivore arthropods did not show significant difference in overall amount of VOC emissions, although MeJA treated trees released several specific compounds which were not found in herbivore-induced trees (Amo et al. (2022)). Although further experiments are needed to find the specific compounds or blends responsible for the attractiveness of predators, our

study indicates that beta-ocimene, and possibly linalool and caryophyllene, might play a significant role.

Overall abundance of insects was marginally significantly higher on English oak in comparison to sessile oak. In addition, analyses of individual arthropod guilds shown that numbers of predators and sap suckers were significantly higher on English oak. English oak is a dominant species and the third most abundant tree of European deciduous forests (Eaton et al., 2016), which hosts a high diversity of specialist and generalist herbivorous insects (i.e., leaf chewers, gall-inducers, and leaf rollers; Southwood et al., 2005; Moreira et al., 2018), while sessile oak is not well explored in the context of insect communities. Moreover, sessile oak grows on drier and warmer sites with acidic soils (Röhrig et al., 2020) that support denitrification of soil (Šimek et al., 2002) and thus influence the plant quality in general and nutrient supply. Thus, the soil contents influence secondary metabolites and overall defensive strategy of the plant.

MeJA treatment had no effect on mean abundance of arthropods on any of the two species. However, application of MeJA had a significant effect on mean body size of individual feeding guilds. Specifically, on both oak species, the mean body size of the assembly of chewers was smaller after MeJA application. This result agrees with current knowledge of effect of plant nutrient supply, secondary metabolites content and high amounts of defensive compounds on the caterpillar growth (Coley et al., 2006). Alternatively, MeJA attracts predators that preferentially feed on larger prey, so the overall mean body size of herbivorous insects surviving on a MeJA-treated plant is smaller. The mean body size of sap suckers and other arthropods in English oak and predatory and other arthropods in sessile oak has increased. Application of MeJA had no effect on mean body size of

predatory arthropods for English oak, and of sap suckers for sessile oak. These results are, for its inconsistency, likely not ecologically important and further, more detailed tests would be needed to resolve the effect of MeJA on the size of insects.

Arthropod predators were the most abundant guild collected from the studied trees and represented about 63.3 % of all arthropods. Interestingly, control trees hosted more mesopredators than the MeJA-treated trees. Greenhouse experiments on *Ficus* trees showed, oppositely, that predatory arthropods were more abundant on MeJA-treated trees in contrast to controls (Xiao et al., pers. communication). Predaceous arthropods are often hunted by larger predators (Serée et al., 2021). Thus, it is likely that invertebrate predators in our experiment avoided MeJA-treated trees due to a potentially higher chance of attack from larger predators or alternatively, were already eaten by bigger predators prior the sampling.

MeJA-treated trees of both oak species hosted higher abundances of sucking insects than control trees. Considering that sucking insect was represented mostly by aphids, these results are in contrast with previous studies (Zhu-Salzman et al., 2004; Brunissen et al., 2010; Cao et al., 2014). For example, aphids (*Schizaphis graminum*) were repelled by MeJA-treated great millet (*Sorghum bicolor*) (Zhu-Salzman et al., 2004). Similarly, Cao et al. (2014) showed that aphids (*Sitobion avenae*) were more attracted to Common wheat (*Triticum aestivum*) without MeJA spraying. In contrast, application of MeJA had no significant effect on the same aphid species in experiments of the same author on Chinese cabbage (*Brasica pekinensis*) (Cao et al., 2016). In our experiment, ants, particularly the genera *Lasius* and *Myrmica*, built nests in five out of 60 of our pots and

reared aphids in higher abundances which might affect the results of insect communities. However, the ant nests were built on trees regardless of treatment and species.

In summary, we found that chemical inducible defence differs between two closely related oak species (English oak and sessile oak). Further, we showed that volatile emissions of defending oak species increase the predation and change insect communities. Specifically, the total mean amount volatiles emitted by MeJA-induced trees differed among the experimental tree species over the time and multiple applications of MeJA. In both plant species, predation was significantly higher on MeJA-treated trees than on control trees. Birds were responsible for the majority of attack attempts, followed by ants and other arthropods. MeJA application had no significant effect on abundances of insect. However, the mean body size of chewers was affected by MeJA treatment negatively.

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Supplementary material

QP_Con_1	QP_MeJA_1	QR_Con_1	QR_MeJA_1	QP_Con_2	QP_MeJA_2
QR_MeJA_2	QR_Con_2	QP_MeJA_3	QP_Con_3	QR_MeJA_3	QR_Con_3
QP_Con_4	QP_MeJA_4	QR_Con_4	QR_MeJA_4	QP_Con_5	QP_MeJA_5
QR_MeJA_5	QR_Con_5	QP_MeJA_6	QP_Con_6	QR_MeJA_6	QR_Con_6
QP_Con_7	QP_MeJA_7	QR_Con_7	QR_MeJA_7	QP_Con_8	QP_MeJA_8
QR_MeJA_8	QR_Con_8	QP_MeJA_9	QP_Con_9	QR_MeJA_9	QR_Con_9
QP_Con_10	QP_MeJA_10	QR_Con_10	QR_MeJA_10	QP_Con_11	QP_MeJA_11
QR_MeJA_11	QR_Con_11	QP_MeJA_12	QP_Con_12	QR_MeJA_12	QR_Con_12
QP_Con_13	QP_MeJA_13	QR_Con_13	QR_MeJA_13	QP_Con_14	QP_MeJA_14
QR_MeJA_14	QR_Con_14	QP_MeJA_15	QP_Con_15	QR_MeJA_15	QR_Con_15

Figure S1: Arrangement of experimental trees across the study site. We arranged trees regularly starting from the first row with sessile oak (QP) control (Con), MeJA-treated (MeJA) sessile oak (QP), English oak (QR) control (Con), MeJA-treated (MeJA) English oak (QR), etc., to cover the entire study area.

S2: Analysis of plant volatiles (according to Amo et al. 2022):

Before the experiment, TD tubes were conditioned with a gas chromatograph (5890 Agilent, modified for spike and cleaning TD tubes) for 30 min at 320 °C and a Helium flow rate of 20 mL/min at the Institute of Environmental Technology in Ostrava-Poruba, Czech Republic. The induced volatile samples were analyzed with a gas chromatograph–mass spectrometer (Agilent, GC 7890 + MSD) at the Institute of Environmental Technology in Ostrava-Poruba, Czech Republic. Trapped compounds were desorbed with two-stage thermal desorption using a thermal desorption unit (Perkin-Elmer Turboamatrix TD 300) under described temperatures: Valve = 200 °C, Primary desorption = 250 °C 5 min⁻¹, Trap = -10 °C, Secondary desorption = 300 °C min⁻¹, Transferline = 200 °C; and flows: Desorption

= 40 mL min⁻¹, Intel Split = 0 mL min⁻¹, Col. = 1 mL min⁻¹, Out Split = 10 mL min⁻¹; Total split = 9.1%).

Desorbed analytes were injected onto an HP-5 capillary column (30 m, 0.25 mm, 0.25 μm film thickness, Hewlett-Packard) with helium (5N) as a carrier gas with a flow rate of 1 ml min⁻¹. The oven temperature program was held at 60 °C for 2 min, then raised to 120 °C at a rate of 10 °C min⁻¹, and finally on to 250 °C at a rate of 30 °C min⁻¹ with a 5 min delay. The compounds (mono-, homo- and sesquiterpenes, and green leaf volatiles (GLVs)) were identified by comparing their mass spectra with those in the pure standards. Pure chemicals were supplied by Sigma-Aldrich and Supleco, prepared by weighing into methanol:

(1) Cannabis terpene Mix B (CRM40937 Supleco, 2000 μg/mL of each component):

Limonene (cyclohexene, 1-methyl-4-(1-methylethenyl)-), C₁₀H₁₆, CAS 138-86-3; β-pinene (bicyclo[3.1.1]heptane-6,6- trimethyl, 2-methylene), C₁₀H₁₆, CAS 127-91-3; β-Caryophyllene (trans-(1R,9S)-8-Methylene-4,11,11-trimethylbicyclo[7.2.0]undec-4-ene), C₁₅H₂₄, CAS 87-44-5; Phytol (3,7,11,15-Tetramethyl-2-hexadecen-1-ol), C₂₀H₃₉O, CAS 7541-49-3; Geraniol (trans-3,7-Dimethyl-2,6-octadien-1-ol), C₁₀H₁₈O, CAS 106-24-1; (1S)-(-)-Camphor ((1S)-1,7,7-Trimethylbicyclo[2.2.1]heptan-2-one), C₁₀H₁₆O, CAS 464-48-2; Terpinolene (p-Menth-1,4(8)-diene), C₁₀H₁₆, CAS 586-62-9; β-Eudesmol ((2R,4aR,8aS)-Decahydro-8-methylene-α,α,4a-trimethyl-2-naphthylmethanol), CAS 473-15-4; (+)-Borneol (endo-(1R)-1,7,7-Trimethylbicyclo[2.2.1]heptan-2-ol), C₁₀H₁₈O, CAS 464-43-7; cis-Nerolidol (3,7,11-Trimethyl-1,6,10-dodecatrien-3-ol), C₁₅H₂₆O, CAS 7212-44-4; α-Terpineol (2-(4-Methylcyclohex-3-en-1-yl)propan-2-ol), C₁₀H₁₈O, CAS 98-55-5; (1S)-(+)-3-Carene ((1S)-3,7,7-

Chapter IV

Trimethylbicyclo[4.1.0]hept-3-ene), $C_{10}H_{16}$, CAS 498-15-7; Linalool ((±)-3,7-Dimethyl-3-hydroxy-1,6-octadiene), $C_{10}H_{18}O$, CAS 76-70-6; p-Cymene (1-Isopropyl-4-methylbenzene), $C_{10}H_{14}$, CAS 99-87-6.

(2) Cannabis terpene Mix A (CRM40755 Supleco, 2000 $\mu\text{g/mL}$ of each component):

α -Pinene (2,6,6-Trimethylbicyclo[3.1.1]hept-2-ene), $C_{10}H_{16}$, CAS 80-56-8; Camphene (3-methylidenebicyclo[2.2.1]heptane), $C_{10}H_{16}$, CAS 79-92-5; β -Myrcene (7-Methyl-3-methylideneocta-1,6-diene), $C_{10}H_{16}$, CAS 12-35-3; 3-Carene (3,7,7-Trimethylbicyclo[4.1.0]hept-3-ene), $C_{10}H_{16}$, CAS 13466-78-9; D-Limonene (1-Methyl-4-(prop-1-en-2-yl)cyclohex-1-ene), $C_{10}H_{16}$, CAS 5989-27-5.

(3) Single chemicals (Sigma-Aldrich):

Caryophyllene oxide, CAS 1139-30-6; Ocimene (3,7-Dimethyl-1,3,6-octatrien), $C_{10}H_{16}$, CAS 13877-91-3; cis-3-hexenyl Acetate, $C_8H_{14}O_2$, CAS 3681-71-8; Methyl Jasmonate (Methyl 3-oxo-2-(2-pentenyl)cyclopentaneacetate), $C_{13}H_{20}O_3$, CAS 39924-52-2. Emissions were presented qualitatively.

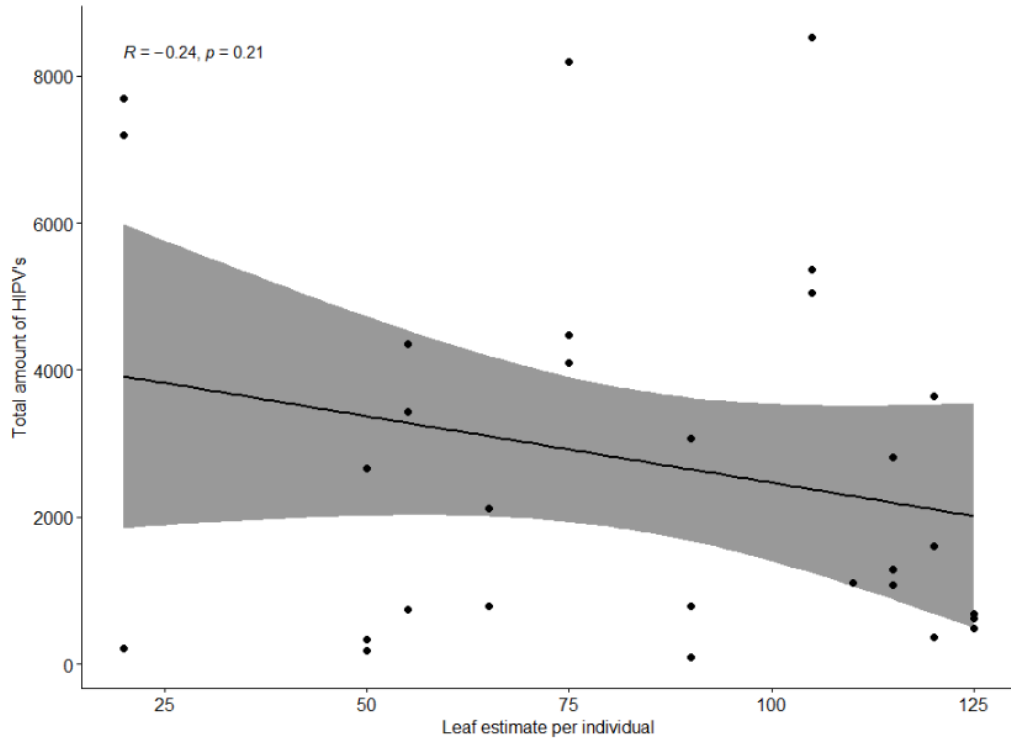


Figure S3: Total amount of sampled HIPVs did not correlate with the number of leaves growing on both species. Tested by the method of Pearson's correlation ($R = -0.24, p = 0.21$).

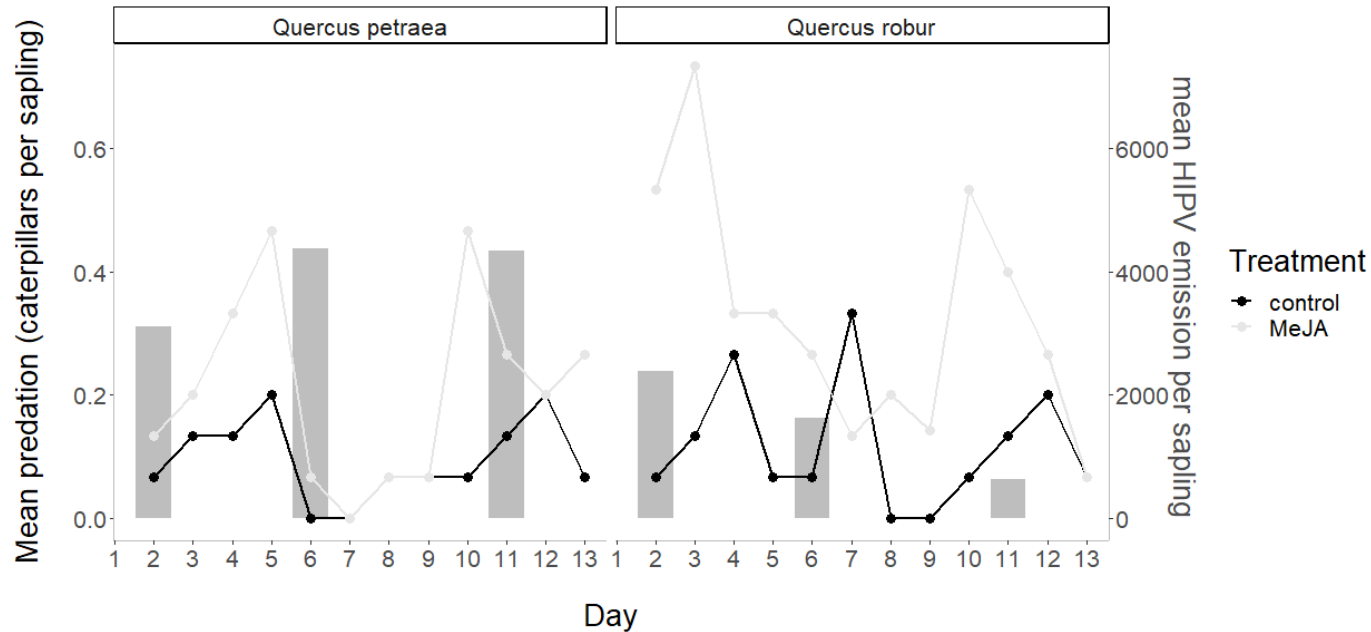


Figure S4: The illustration of predation rate and defensive compounds emission throughout the course of 13 days long experiment. In both oak species, the predation rate decreased from 6th to 9th day of the experiment and increased again to the initial level. While for sessile oak (*Quercus petraea*) total amounts of defensive compounds were increasing, English oak (*Quercus robur*) showed an opposite trend.

Chapter V

Naïve Blue tits (*Cyanistes caeruleus*) and Great tits (*Parus major*) prefer to search for food in herbivore damaged than in undamaged shrubs

Anna Mrázová and Kateřina Sam

Naïve Blue tits (*Cyanistes caeruleus*) and Great tits (*Parus major*) prefer to search for food in herbivore damaged than in undamaged shrubs

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Abstract

The olfactory sense in birds is a phenomenon examined for almost 60 years now. Recently, however, research has focused on the use of olfactory by insectivorous birds, along with vision, while foraging for insect prey. It is well known that herbivore-attacked plants release herbivore-induced plant volatiles (HIPVs), which could be used as a cue by insectivores. However, the results of existing studies focusing on the importance of visual and chemical cues for birds searching for prey are contradictory. The aim of our study was to test whether Great Tits (*Parus major*) and Blue Tits (*Cyanistes caeruleus*) use chemical or visual signals (i.e., herbivory damage) to locate herbivore-rich trees. We used caterpillars of ailanthus silkmoth (*Samia cynthia walkeri*) and European privet (*Ligustrum vulgare* ‘Atrovirens’) as our study system. On the privets, four treatments were prepared, which emitted (1) visual signals (i.e., visually damaged leaves by chewing herbivores, but no HIPVs), (2) chemical signals (i.e., HIPVs but no visual signal), (3) a combination of signals (visual and HIPVs) and (4) no signal (control). We studied the attractiveness of these four treatments for the tits in two pair-choice aviary tests. Both bird species preferred to search on shrubs emitting (1) a combination of visual and chemical signals over control trees providing no signal, (2) a chemical signal over a visual signal.

Introduction

Birds are important predators of herbivorous insects and effective restrictors of the damage they cause to plants (e.g., (Van Bael et al., 2003, Mooney et al., 2010, Maas, 2013, Maas et al., 2015), thus having an effect on ecosystem functioning (Metcalf et al. 2014). Birds orient themselves mostly visually, and their superb eyesight is essential, especially while feeding (Emery, 2006, Shimizu et al., 2010). The olfactory sense was described in birds about 55 years ago (Wenzel, 1967), but Mäntylä et al. (2004) suggested, for the first time, that insectivorous birds may use it also while searching for insects. To this day, it remains unclear how birds combine or prioritize visual and olfactory information when making foraging decisions.

Insects try to be visually unobtrusive (Lev-Yadun & Inbar, 2002) and odourless (Schröder & Hilker, 2008), in the hope of escaping the attention of insectivorous birds. However, they leave behind unavoidable visual signs of their presence. Excrement, chewing damage (Sam et al., 2015), or leaf rolls in which they hide (Vieira & Romero, 2013, Sam et al., 2014), might be perceived by birds as a sign of insect presence (Boege, 2006, Müller et al., 2006). The presence of insects on plants may also be revealed by more subtle visual signals. Due to the negative effect of herbivory on photosynthesis, insect infested leaves were shown to have lower reflectance, which can be detected by birds (Mäntylä et al., 2008, Amo et al., 2013). Naturally, plants would benefit from providing structural, visual (Marquis and Whelan 1996) or chemical signals which can be attractive to the predators in revealing the presence of herbivorous insects (Turlings et al., 1995). Herbivore-induced plant volatiles (HIPVs), inducible volatile compounds emitted after herbivorous damage, serve this

purpose, as plants often emit a wide variety of them in response to herbivorous insect attack (Mumm and Dicke, 2010; Dudareva et al., 2006; Peñuelas & Llusà, 2004; Turlings & Wäckers, 2004; Kessler and Baldwin, 2001; Knudsen & Tollsten, 1993).

The first successful attempt to separate the bird's reaction to the chemical and visual signal provided by plants (in terms of change in reflectance due to changes in rate of photosynthesis) was conducted on trained Great tits searching for food on apple trees (*Malus sylvestris*). The birds preferred to search apple trees that transmitted only chemical signals (HIPVs) rather than on trees providing visual signals (lower reflectance) (Amo et al., 2013; Fig. S3). Furthermore, the same team showed that naïve Great tits were not able to distinguish between herbivore-free and herbivore-infested trees providing the birds both with chemical and visual signals (Amo et al., 2016; Fig S3). To the best of our knowledge, the response of birds to actual herbivory damage caused by chewers has not been tested with response to chemical signals provided by trees yet.

It is known that hatchlings learn the appearance of suitable insect prey from parents bringing food to the nest (Arnold et al., 2007). Since it was implied that Great tits need to learn the chemical cues or subtle visual cues of the insect's presence on plants (Amo et al., 2016), we were wondering whether tits need to learn also other sign of the presence of the prey, or whether they have this ability innate. Firstly, we aimed to test whether birds have an innate ability to distinguish between undamaged control shrubs and shrubs that have obviously damaged leaves, and which are simultaneously providing a chemical signal in form of HIPVs. In the second experiment, we aimed to test the response of birds on visually damaged leaves and their HIPVs signal. We further hypothesised that the

behaviour of two tested bird species (Great tits and blue tits) would not differ in response to the signals. Despite the lack of experimentation with Blue tits, they were expected to search for prey similar way as Great tits, which were employed more often (Mrazova et al., 2019).

Materials and methods

We carried out the behavioural experiments in a calm part of the campus of the Faculty of Science, University of South Bohemia in Ceske Budejovice, Czech Republic (48.9769419N, 14.4449758E, 375 a.s.l.). All tests were performed in an aviary which consisted of an entrance area (1,5 x 1,5 x 2 m) and an experimental area of trapezoid shape (1,5 x 2,5 x 3 x 2,5 m; Fig. S1). Two identical sheets of opaque, breathable fabric (1.5 x 2 m; Fig. S1) were placed permanently on both sides of the aviary throughout the entire study (*Experiment 1 and 2*). We conducted all the experiments between 15th and 21st of June 2016.

Birds

We used two locally common insectivorous birds – Great tits (*Parus major*) and Blue tits (*Cyanistes caeruleus*). Since we aimed to study the reactions of naïve birds to the various signals from plants, we used hand-raised birds. We collected a total of 30 hatchlings from the nest-boxes on the 5th and 6th of May 2016 in nearby Bor forest (48.9815919N, 14.4219594E). We ringed all individuals (Great tit N = 18, Blue tit N = 12; AM; Licence ID: 1152, The Ringing Organisation of National Museum in Prague) and placed them in cages (40 x 40 x 60 cm) in the accredited breeding room of the Faculty of Science. The study was carried out based on a licence issued by the Ministry of the Environment of the Czech Republic under the Law on the Protection of Experimental Animals (Ref.

75779/ENV/15-3418/630/15). We kept all hatchlings collected from a given nest (2-3 individuals per nest) together in one cage until fledged. The birds were kept under natural daylight (5:15 to 20:30) augmented with fluorescent light tubes and automatically opening and closing window shades. During this period, hatchlings were individually hand fed every 30 min (between 6AM and 6PM) with hand rearing food: the mixture consisted of mealworms (*Tenebrio molitor*), boiled eggs, carrots, curd, dry insect mixture and vitamins (VERSELE-LAGA OROPHARMA Omni-vit), all mixed together in a food processor. Hand feeding continued after fledging, but the intervals were prolonged to 1h and later to 4h, to stimulate independent feeding of birds from bowls with food provided ad libitum. When independent, about 35 days after hatching, birds were placed to the cages individually. From then on, sprouted mixed seeds, commercial dried insects (Nobby Orlux Insect Patee Premium) and water enriched with vitamins (VERSELE-LAGA OROPHARMA Omni-vit) were provided ad libitum. Mealworms were offered only as supplementary food (three mealworms × four times a week).

Plants and insects

We selected European privet (*Ligustrum vulgare* 'Atrovirens'; L., 1753) and Ailanthus silkmoth (*Samia cynthia walkerii*; Felder, 1862) for our study system. We used the European privet for its availability, rapid growth, and suitability as a host plant for several larvae of Lepidoptera species. The European privet is a relatively common shrub in the biotope of our experimental birds. For the experiment, we planted 15 individuals of ca. 1.5 m high privet in 30 litre plastic pots. Caterpillars of Ailanthus silkmoth (2nd instar) were used to prepare treatments, i.e., upregulate the production of herbivore-induced volatiles in our plants. The silkmoth is

indigenous in Asia and introduced to many central and southern European countries (e.g., Germany, Austria, Hungary). Thus, used caterpillars are a part of the diet of both experimental bird species in areas where they occur simultaneously (including neighbouring countries of the Czech Republic). We selected the silkmoth for its commercial availability and the willingness of tits to feed on younger instars. We bred ca. 200 individuals of caterpillars in the insectarium of the Institute of Entomology, Biology Centre Academy of Science, Czech Republic on branches of European privet at a controlled temperature of 23°C.

Habituation trials

In order to support the bird's natural ability to search for food, normally learnt in the wild, and to avoid a fear of the unknown, all birds underwent habituation trials in a relatively large aviary just after they started to eat independently. We placed two intact shrubs of European privet (*Ligustrum vulgare* 'Atrovirens') into the two far corners in aviary (Fig. S1). We attached five live mealworms by entomological pins to the leaves of each of the two privets. Each bird spent two hours a day (a total of 10 hours over the course of 30 days) in this aviary, to learn how to search for exposed food on foliage, and not just in a bowl. After each habituation trial, birds were captured by a net and returned to accommodation cage and the number of consumed mealworms counted. Birds were considered to be habituated and able to search for food, when they removed at least 80 % of exposed mealworms during the most recent habituation trial. The habituation trials are necessary, because without them the birds fly frantically in the novel large aviary and are typically scared of green foliage and thin branches which move when they try to sit on them. Thus, our birds got used to the experimental environment but remained naïve to

herbivore-induced volatile compounds produced by privets after the attack of caterpillars and to herbivory damage on leaves. Shrubs with pinned mealworms were used only for habituation and they were excluded from further experiments.

Preparation of plant treatments

We prepared four plant treatments: **(1) Control** (no treatment) privet shrubs did not provide any signal to birds. During the preparatory phase of the experiment, control shrubs were placed at least 50 m apart from other treatments in order to prevent their induction by any volatile compounds released from plants undergoing herbivorous damage. Leaves of privets providing a **(2) visual signal** (i.e., visible leaf damage) were eaten by caterpillars for 48 hours during which the caterpillars ate about 50% of provided leaf area. To prepare shrubs with the visual signal, we placed the caterpillars (N = 10 per treatment, 2nd instar) individually in clip-cages (Ø 45 mm) surrounding tips of 2-3 branches with at least 30 leaves. Clip-cages delimited an area where caterpillars can eat and prevented their escape (Amo et al., 2013). The caterpillars were then removed, and shrubs were left aside for another 72 hours, so that only the visual signal (i.e., the damaged leaves) was presented to birds at the time of the experiment. Wounds caused by feeding caterpillars are healed and production of volatiles stops or slows down significantly after 72 hours (Schoonhoven et al. 2005). If present, any excrement was swept away with a soft brush. The leaves of privet shrubs providing **(3) both signals** (visual and chemical) were eaten by caterpillars for 48 hours. The caterpillars were removed immediately prior to experiments. Thus, these shrubs provided both visual (i.e., damaged leaves) and chemical (i.e., HIPVs) signal. The shrub providing only **(4) chemical signal** was created as a combination of two

differently treated shrubs. Control shrub (providing no signal) was placed inside of the aviary. A shrub providing both signals (chemical and visual) was placed just behind the opaque permeable fabric (2 x 1.5). Birds could therefore perceive a chemical signal released by shrub placed behind the fabric but had no visual signal; Fig. S2). A chemical signal is usually spread to 2 - 5 m (Sam et al. in prep.) and it is unrecognizable above 10 m (Braasch and Kaplan, 2012).

At this time, it is important to point out that what we call “visual signal” is not comparable with “visual” treatment in the comparable study by Amo et al. (2013). While we refer to visible herbivory damage caused by chewing damage, Amo et al. refer to visible changes in terms of leaf reflectance (Amo et al., 2013). Further, what we call “both signals” in the current study, corresponds to “damaged leaves” in their study. “Chemical” signals are identical in both studies (Fig. S3).

Experimental setup

Prior to the experiments, each individual bird was starved for 1.5 hours in their cage in the breeding area, placed into a cotton bag, and released into the outdoor experimental aviary. The study consisted of two independent pair-choice experiments which lasted 30 minutes each (including 5 minutes for calming down). In *Experiment 1*, birds were offered to choose between control shrubs providing no signal and shrubs providing both signals (visual and chemical). *Experiment 2* was a pair-choice between shrubs providing only visual or only chemical signal. Each bird was used only in one test in a day, but each bird was used in both tests by the end of

the experimental season. In contrast to habituation trials, birds were not offered food rewards during the experiments.

The two experimental shrubs were placed in the aviary just prior to each test. Their location (left or right corner) in the aviary was chosen randomly. The order of experiments and their tests was also chosen randomly for each bird individual. We aimed to conduct all tests during warm summer days (average temperature measured in aviary = $31\text{ }^{\circ}\text{C} \pm 3^{\circ}\text{C}$) from 9 AM to 3 PM. However, we were not able to avoid one shower (i.e., we were not able to synchronize the preparedness of birds, caterpillars, and unexpected change of weather at the same time). It is known that temperature, precipitation and humidity have a significant effect on the amount of volatile compounds emitted (Vallat et al., 2005). As the aviary was roofed, the rain itself should have no significant effect on our results, and the temperature did not decrease by more than 7°C during the day with shower.

The behaviour of the birds in the aviary and their foraging behaviour on shrubs during all tests was recorded by digital camera Panasonic HC-V510EP-K. Later, we analysed three behavioural parameters: (1) first choice (i.e., the signal for which the birds decided to land as soon as they were released to aviary), (2) number of visits on individual shrubs, and (3) time spent searching for food on individual shrubs and thus the signal provided by them. Immediately after each test, the bird was captured by a net in the aviary and returned to the cages in breeding area where water and food was offered to it ad libitum again.

Collection of plant volatiles

We collected plant volatiles from a subset of the differently treated shrubs after the first experimental day. We collected all samples and blanks (i.e.,

ambient air) simultaneously. To sample the volatiles or blank samples, we enclosed randomly selected branches of selected shrubs in polyethylene terephthalate (PET) bags (size 35 x 9 x 43 cm; Tesco, Polska, Kraków) or we trapped ambient air to empty bags. Bags were loosely fastened using a cable tie to allow some air flux along the branch (or without the branch – blank samples). After 20 minutes, we cut one outward-facing corner of the bag and inserted a glass tube for thermal desorption (TD tube) containing 150 mg of Tenax TA adsorbent (Supelco, mesh 60/80; Bellefonte, PA, USA). The glass tube was connected by a silicon tube to a vacuum pump (Spectrex, PAS-500, Redwood City, California, USA) which sucked air from the bag (through adsorbent). Air was sucked at a flow rate of 200 ml/min (Mrazova and Sam 2018). Active air sampling was conducted for 30 min and a total of 6 l of air thus went through each TD tube. After sampling, the TD tubes were collected, closed with silicon caps, and kept refrigerated at 4°C for about 1 week before analysis by gas chromatography–mass spectrometry.

Analysis of plant volatiles

The collected volatile samples were analysed by a gas chromatograph–mass spectrometer (Hewlett-Packard GC 6890, MSD 5973; Palo Alto, CA, USA). Trapped compounds were desorbed with a thermal desorption unit (Perkin-Elmer ATD400 Automatic Thermal Desorption system) at 250 °C for 10 min, cryofocused at 230 °C and injected onto an HP-5 capillary column (50 m 60.2 µm i.d. 60.5 mm film thickness, Hewlett-Packard) with helium as a carrier gas. The oven temperature programme was maintained at 40 °C for 1 min, then raised to 210°C at a rate of 5°C min⁻¹ and finally on to 250°C at a rate of 20°C min⁻¹. The compounds (mono-, homo- and sesquiterpenes and green leaf volatiles, GLVs) were identified by

comparing their mass spectra with those in the pure standards. Pure chemicals were supplied by Sigma-Aldrich, prepared by weighing into methanol: caryophyllene oxide, CAS 1139-30-6; ocimene, CAS 13877-91-3; cis-3 hexenyl acetate, CAS 3681-71-8; linalool, CAS 76-70-6; isoprene, CAS 78-79-5; α -pinene (bicyclo[3.1.1]hept-2-ene, 2,6,6-trimethyl-), CAS 80-56-8; camphene (bicyclo[2.2.1]heptane, 2,2-dimethyl-3-methylene), CAS 79-92-5; β -pinene (bicyclo[3.1.1]heptane-6,6-trimethyl, 2 methylene, C₁₀H₁₆) CAS 127-91-3; 3-carene (bicyclo[4.1.0]hept-3-ene, 3,7,7-trimethyl-, C₁₀H₁₆) CAS 13466-78-9; β -myrcene (1,6-octadiene, 7-methyl-3-methylene-, C₁₀H₁₆), CAS 123-35-3 and limonene (cyclohexene, 1-methyl-4-(1-methylethenyl)-, C₁₀H₁₆), CAS 138-86-3. Emissions were presented qualitatively. Analyses were conducted at the Institute of Environmental Technology in Ostrava-Poruba, Czech Republic under a supervision of Mgr. Martina Vráblová (Mrazova and Sam 2018).

Statistical analyses

All statistical analyses were done using R software (version 4.1.2). To analyse bird preferences, we counted (1) a number of visits to individual shrubs and (2) a total time (in seconds) spent on them. Unfortunately, we were not able to analyse the first choice of the birds. Many birds landed first on the netting of the aviary and took differentially long before deciding where to search. Others quickly disappeared to a shrub which did not seem to be an intentional choice.

For the number of arrivals, we fitted a logistic model (estimated using ML) to predict the absolute number of arrivals (response variable) dependency on Species and Side (explanatory variables): `cbind(arrivals on shrubs providing chemical signal, total number of arrivals - arrivals on`

shrubs providing chemical signal) \sim Species * Side. For time spent searching on shrubs, we fitted a beta regression additive model to predict weighted values of time (response variable) dependency on Species and Side (explanatory variables): Proportion of time \sim Species + Side. Throughout the article we report mean values \pm standard error. We plotted percentage values (i.e., variable values used at analyses \times 100) of time spent searching and number of arrivals to each treatment.

Results

Arrivals to shrubs: In total, we recorded 2,214 arrivals to individual shrubs in both experiments (60 tests in total). During *Experiment 1*, both bird species chose the shrubs providing none or both signals equally often (ANOVA $\chi^2 = 0.59$, Df = 1, $p > 0.05$). However, in *Experiment 2*, Great tits arrived at shrubs providing chemical signal significantly more often when the shrub was located on the left side of the aviary (Tab. 1; Fig. 1). Blue tits, on the contrary, preferred the chemical signal provided by a shrub located on the right side of the aviary (Tab 1; Fig. 1).

Table 1: The results of generalized linear model with binomial distribution for *Experiment 2* showed that the number of arrivals to both shrubs providing chemical and visual signal did not differ between the bird species. Significant interaction between the bird species and side of the cage, however, indicated that the Great tits arrived more often to the chemical shrub when it was placed on the left side of the cage, while Blue tits preferred chemical treatment when located on the right side of the aviary. The side of the cage itself did not have any significant effect on the number of arrivals to the shrubs.

	Estimate	Std. Error	z value	Pr(> z)
(Intercept)	-0.1092	0.2701	-0.404	0.686
Species Great tit	0.8468	0.3744	2.262	0.0237 *
Side Right	0.5612	0.4357	1.288	0.1977
Species Great tit:Side Right	-1.2689	0.5628	-2.255	0.0242 *

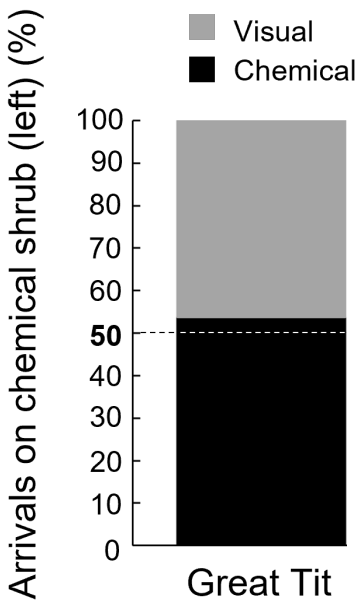


Figure 1: Mean percentage of the number of visits of Great tits (N = 18) to individual shrubs offered in *Experiment 2*. Great tits preferred to search on shrubs providing chemical signal over the shrub providing visual signal only when this shrub was located on the left side of the aviary. The percentage of time spent by searching for food was calculated as: $(\text{time spent on the focal shrub} / (\text{time spent on the focal shrub} + \text{time spent on the other shrub})) \times 100$.

Time spent on shrubs: During all tests, birds spent a relatively short time foraging for food on the shrubs, while they devoted a significant proportion of the experimental time to perching and observing. Altogether,

birds spent 106 out of the 1500 minutes searching for food on individual shrubs (i.e., 30 birds * 25 minutes/test/bird * 60 tests = 1500 minutes in total). Great tits spent an average of 3.94 ± 1.25 of each 25 min long experiment searching, while Blue tits spent 2.91 ± 2.42 minutes searching on shrubs.

In *Experiment 1*, both studied species searched significantly (ca. 5 times) longer on shrubs emitting both signals than on shrubs providing no signal (Tab. 2A; Fig. 2). However, Great tits spent significantly less time searching for food on shrubs providing both signals than Blue tits (Tab. 2A, B). In *Experiment 2*, when the birds were offered shrubs providing chemical and visual signals individually, birds preferred to search the shrubs with the chemical signal (Tab. 3A; Fig. 2). Great tits spent significantly more time in active searching on chemical shrubs than Blue tits (Tab. 3B). Side and side in interaction with bird species had a significant effect on time spent searching on provided treatments. Similarly, as in the analysis of arrivals, Great tits preferred to search shrubs emitting chemical signals only when it was placed on the right side. Experimental day and bird individual identity had no significant effect on the observed results in any of the analyses.

Table 2: The results of generalized linear model with binomial distribution for *Experiment 1* showed that both bird species (A) preferred searching shrubs emitting both signals than on control shrubs. (A) Time spent searching on both shrubs providing no and both signals differed between the bird species. The side of the cage did not have any significant effect on the time spent searching on the shrubs. (B) Blue tits spent more time searching shrubs providing both signals than Great tits.

A)

	Estimate	Std. Error	z value	Pr(> z)
(Intercept)	1.3357	0.1368	9.762	< 2e-16 ***
Species Great tit	-0.7266	0.1505	-4.826	1.39e-06 ***
Side Right	-0.1729	0.1463	-1.181	0.237

B)

Species	emmean	SE	df	asypm.LCL	asypm.UCL
Blue tit	0.777	0.0233	Inf	0.731	0.823
Great tit	0.628	0.021	Inf	0.586	0.669

Table 3: The results of generalized linear model with binomial distribution for *Experiment 2* showed that both bird species preferred searching on shrubs providing chemical signal than on shrubs providing visual signal (A). Significant interaction between the bird species and side of the cage, however, indicated that the Great tits spent more time on the chemical shrub when it was placed on the left side of the cage. The side of the cage itself did also have a significant effect on the number of arrivals to the shrubs. (B) Great tits spent more time searching on shrubs providing both signals than Blue tits.

A)

	Estimate	Std. Error	z value	Pr(> z)
(Intercept)	0.54	0.1674	3.226	0.00126 **
Species Great tit	0.7261	0.2368	3.066	0.00217 **
Side Right	0.6402	0.2537	2.523	0.01163 *
Species Great tit:Side Right	-0.8619	0.3758	-2.293	0.02184 *

B)

Species	emmean	SE	df	asypm.LCL	asypm.UCL
Blue tit	0.698	0.0276	Inf	0.644	0.752
Great tit	0.76	0.0282	Inf	0.705	0.815

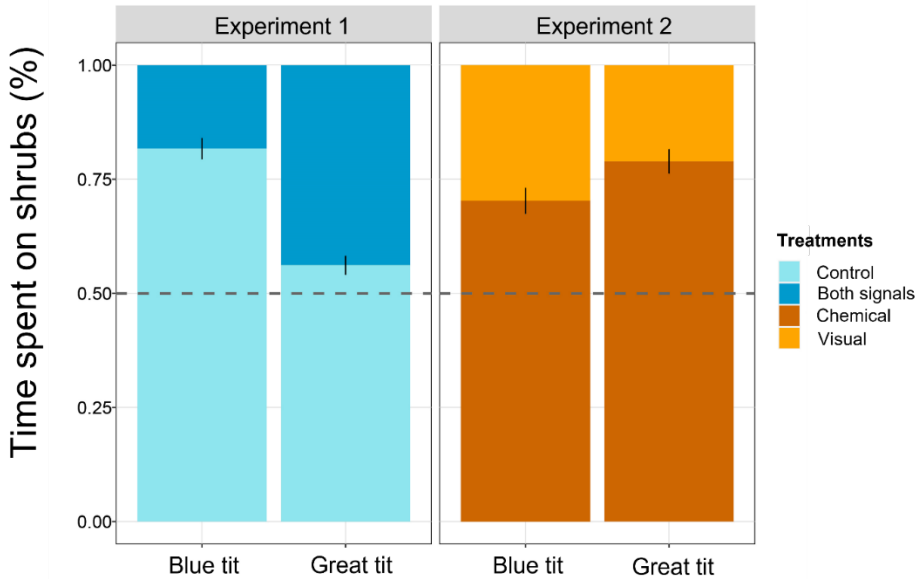


Figure 4: The preference of Great tits and Blue tits towards shrubs providing different signals, estimated by a beta regression model. In the *Experiment 1*, both bird species preferred to search on shrub providing both all signals over the control shrub providing no signal. In the *Experiment 2*, both bird species preferred to search on shrub providing chemical signal over the control shrub providing no signal. The percentage of time spent by searching for food was calculated as: $(\text{time spent on the focal shrub} / (\text{time spent on the focal shrub} + \text{time spent on the other shrub})) \times 100$.

The analyses of volatile compounds proved that volatiles of shrubs, which were supposed to provide only visual signal, indeed did not release different or higher amounts of volatile compounds than control shrubs. On the contrary, shrubs that were supposed to be providing chemical signal released for example benzyl acetate which was not detected in any other treatments of the shrubs. Chemically active shrubs also emitted higher amounts of D-limonene and limonene-oxide than control shrubs and shrubs providing only visual signal (Tab. 4). The production of α -pinene, β -pinene and 3-carene did not differ between the shrubs which were supposed to provide chemical signal and control shrubs (Tab. 4). It is

important to note that this analysis focuses selected compounds. It is therefore possible that the used treatments differ in other, unanalysed compounds.

Table 4: The list of the areas of the individual peaks identified in the individual treatments. The areas are evaluated from chromatograms on selected ions ($A(x) \cdot 10^{-6}$) and comparable between samples, but not even relatively between compounds (the relative abundance of the fragment is characteristic for each sample). RT = retention time.

Treatment		α -pinene	β -pinene	3-carene	D-limonene	benzylalcohol	α -campholenal	limonene-oxide	benzylacetate	pinocarvone
	RT(min)	5,53	6,17	6,667	6,961	7,354	8,508	8,732	8,95	9
Visual	$A(93) \cdot 10^{-6}$	61,8	24,2	23,8	96,2					
	$A(108) \cdot 10^{-6}$					311,9	57,8	4,83		113,01
Control	$A(93) \cdot 10^{-6}$	51,9	29,2	25,94	136,2					
	$A(108) \cdot 10^{-6}$					325,3	64	8		131
Chemical	$A(93) \cdot 10^{-6}$	46,3	16,3	22	161,1					
	$A(108) \cdot 10^{-6}$					380,5		68,6	44,87	

Discussion

We confirmed our hypothesis that naïve Great and Blue tits prefer to search food on shrubs that emit both chemical and visual signals rather than on control shrubs, which give no signal. It suggests that the ability to search for prey according to some cues is innate. To our surprise, we showed that naïve tits prefer to search on shrubs emitting chemical signals rather than on the shrubs providing only visual stimuli in the form of herbivore damage. This would imply that naïve tits are able to use olfaction in the search for insect prey, and our results would thus contradict those of Amo in 2016 (Fig. S3) and Sam et al., 2021.

The level of naivety of the experimental birds proved to be of considerable importance in aviary pair-choice experiments, during which

the senses of the birds are tested. On the other hand, complete naïve birds are difficult to work with, as they are wary of foliage, and they are unfamiliar with the concept of feeding from places other than the provided bowls. Thus, some habituation to the novel experimental environment is typically needed. Our study birds were naïve, hand-raised, and young (12 weeks), but they were habituated to forage on shrubs which provided them with food rewards without being able to associate the reward either with herbivore-induced chemicals, or with visual signal in terms of herbivory damage. Thus, they were naïve to the signals offered in the tests, but not to the environment of the aviary and to the experimental shrubs. From this perspective, our birds were less naïve than those from previously published work of Amo et al. (2016) and Sam et al. (2021), but more naïve than those used by Amo et al. (2013). The Great tits tested in Amo et al. (2013) were one year old, kept in captivity and hand-raised since 10 days old. To habituate them and maintain the birds' interest in searching for larvae during the trials, Amo et al. (2013) allowed the birds to feed on real larvae feeding on trees in the aviary. Thus, their birds obtained experience with both foliage and herbivore induced volatiles and could learn to associate the smell of leaves with food during their five habituation trials. In contrast, in Amo et al. (2016), the birds underwent habituation trials in a tree-free aviary. The Great tits tested in Sam et al. (2021) were collected from nest boxes when they were 10-13 days old. Approximately 35 days after hatching, two thirds of the birds underwent long-term habituation/learning trials while one third was kept naïve, habituated in an aviary with two dead pedunculate oaks (*Quercus robur*) and pinned mealworms at each side of the aviary. In conclusion, it seems that completely naïve birds (Amo et al., 2016, Sam et al., 2021) are not able to

distinguish between the offered signals. However, even very limited experience with foliage (current study), more intense habituation (Amo et al., 2013), or even intended training (Sam et al., 2021), enables the birds to react to chemical signals from herbivore-induced trees effectively.

Existing studies differ in the signals offered. In Amo et al. (2013), experiment 1, the birds were offered a (i) tree with caterpillars as well as visual signal (damaged leaves) and chemical signal (HIPVs), (ii) tree providing visual (damaged leaves) and chemical (HIPVs) signals (iii) tree from which the parts of damaged leaves were removed, so the tree was chemically active, the reflectance was lower, but damage was not visible to birds, (iv) control trees with no damage. Our current *Experiment 1* was thus directly comparable with their experiment testing the preference of treatments (ii) and (iv). Not surprisingly, in both studies, birds preferred to search on trees/shrubs providing both visual and chemical signals rather than on control shrubs/trees. This result is consistent, irrespective of their naivety and the age of tested birds. Considering our birds naivety to the signals, this confirms that even naïve birds have the innate ability to preferentially search on damaged leaves.

In her second experiment, Amo (2013) offered a control tree with no signals and a tree with one of the following signals: (a) chemical (i.e., HIPVs), (b) visual (i.e., change in reflectance), and (c) chemical and visual (i.e., HIPV and change in reflectance). Thus, our second experiment is not directly comparable with these tests, as we compared the reactions of birds to chemical signal (i.e., HIPVs) and to visually detectable herbivory damage directly. Irrespective of the type of visual signal offered, both experiments showed that birds preferred to search on the tree providing chemical cues rather than on the tree providing visual cues. This may imply

that birds consider a specific HIPVs as an indicator of the presence of the food, as long as they had previously some experience with the smell of foliage. Despite our birds lack of direct experience with HIPVs, they previously search for food in foliage, which always releases some volatile compounds. We can hypothesise that the increased amounts of some of these compounds may be attractive to birds. For example, we could imagine that the birds made a positive association with the normal levels of D-limonene during the habituation trials, and that increased amounts of D-limonene were for them even more attractive in tests. Unfortunately, our targeted analysis revealed only increased production of D-limonene and limonene-oxide, but it is likely that herbivore attack upregulated also production of other compounds.

In an ecological context, the ability to react to HIPVs is advantageous. An antipredator strategy of herbivorous larvae includes night feeding on leaves, and leaving the area of damaged leaves before sunrise, to reduce the chances of predation (Seifert et al., 2016). The chemical signal could therefore serve as an indicator of insects currently feeding in the area.

In our study, during the *Experiment 1*, both studied species arrived to offered treatments equally often. In the *Experiment 2*, Great tits arrived at chemical shrubs more often when placed on the left side of the aviary, while Blue tits preferred chemical signals when placed on the right side of the aviary. Our results thus match previously observed behaviour of Great tits tested in similar study (Amo et al., 2016). The birds did not visit the caterpillar-treated apple trees more frequently than the control trees. The results of Amo et al. (2013), on the other hand, showed that Great tits arrived at apple trees providing chemical signals (vs. control), but not at

trees providing visual signals (changes in leaf reflectance; vs. control). The mentioned studies differed in naivety and age of studied birds. Given the inconsistency between our results and previous results, we decided to neglect arrivals as a proxy of the attractiveness of the signals provided.

The tested bird species differed in the strength of response to the offered signals. While during the Experiment 1 Blue tits spent more time searching on shrubs providing both signals than Great tits. Great tits spent significantly more time by searching on chemical shrubs than Blue tits (Tab. 3B). However, both tested species significantly preferred both signals in *Experiment 1* over control and chemical signal over visual signal in *Experiment 2*. Thus, we consider those differences between the reactions of species marginal with no ecological importance.

The location of shrubs in the aviary (left or right side) affected (i) the arrivals of Great tits in *Experiment 2*, left side; (ii) the arrivals of Blue tits in Experiment 2, right side; (iii) the time spent searching of Blue tits in *Experiment 1*, left side. Given the random nature of these results, we do not consider them noteworthy. Although the location of the aviary was chosen within a quiet environment of the campus, it is important to note that the surrounding was not symmetrical. There was a parking lot on the left side of the aviary (ca. 20 m away), and a dirt path used occasionally for dog walking was located behind a fence on the right (ca. 30 m away, Fig. S4). As we are not able to explain the observed statistical preferences to the sides of the cage, we recommend choosing an experimental site with special attention to an ultimately quiet environment in future research, as birds are shy animals, which might react to various subtle signals during behavioural experiments.

In summary, our study showed that naïve Great and Blue tits can be guided by signals emitted by caterpillar-induced plants. Specifically, both species preferred searching for food on privets emitting visual and chemical signal than on shrubs emitting no signal. Further, for the first time, we showed a reaction of Great and Blue tits on isolated chemical and visual (visible herbivorous damage) signal. Both species preferred searching for food on chemically active shrubs, which implies innate ability of naïve birds to use chemical cues when searching for food.

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Supplementary material

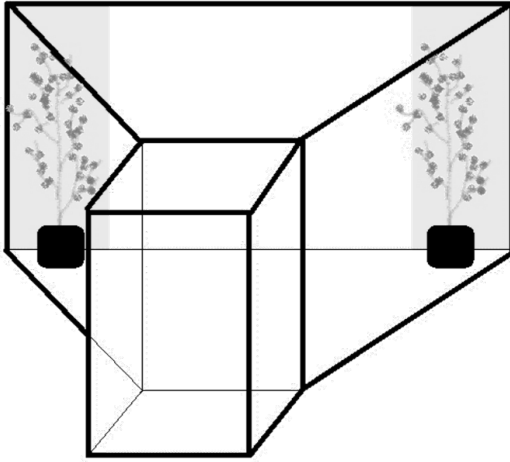


Figure S1: An aviary consisted of an entrance and manipulation area (1,5 x 1,5 x 2 m), and intrinsic test area of trapezoid shape (1,5 x 2,5 x 3 x 2,5 m). Two identical opaque breathable (2 x 1.5 m) cloths were placed at both sides of the aviary throughout both experiments (and used as a part of chemical treatment).



Figure S2: Visualization of chemical signal treatment. The black shrub (in the foreground) was a control shrub (providing no signal) located inside the aviary and therefore visible to the birds. The grey shrub (in the background) providing both signals (visual and chemical) was located outside the aviary behind an opaque permeable fabric and therefore could be smelled but not visible to the birds. The branches of both shrubs were touching the fabric.

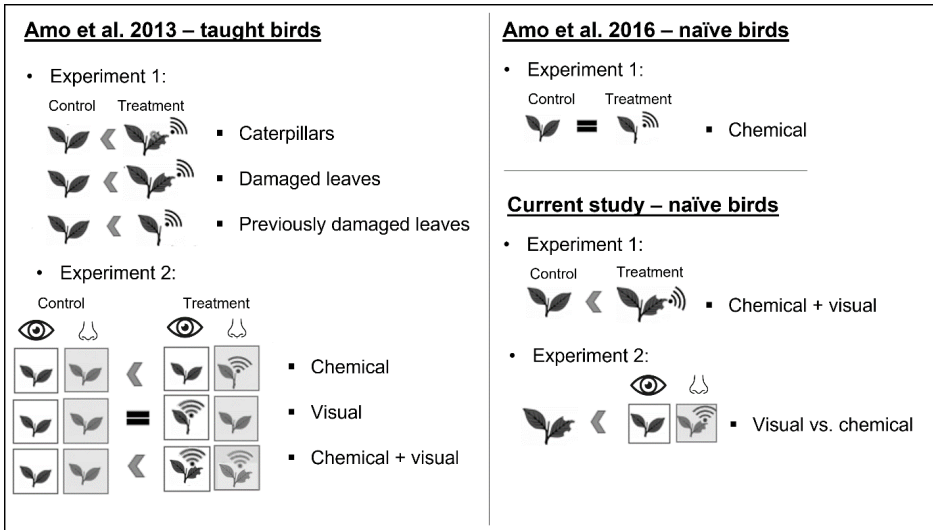


Figure S3: The experimental design and main results of Amo et al., 2013, 2016 and current study. Results of Amo et al., 2013 show that experienced Great tits were attracted to treated in contrast to intact trees, even when they could not see the larvae or their feeding damage. Results of Amo et al., 2016 show that naïve great tits were not attracted to herbivore-damaged trees, when they could not see the larvae or their feeding damage. Results of our current study show that naïve Great and Blue tits prefer to search on shrubs providing (1) chemical and visual signal rather than on the shrub providing no signal (2) chemical signal rather than on the shrub providing visual signal. Legend: eye = what birds can see; nose = what birds can smell. Note that Visual signal of Amo et al., 2013 indicates a tree providing chemical signal and visual signal (lower reflectance) while visual treatment in current study indicates a shrub with damaged leaves but no chemical signal.

Chapter VI

Application of methyl jasmonate attracts predators to conspecific neighbouring Oaks, but not to trees of a sister species.

Kateřina Sam, Leonardo Ré Jorge, and Anna Mrázová

Application of methyl jasmonate attracts predators to conspecific neighbouring Oaks, but not to trees of a sister species.

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Abstract

Plant-plant communication and communication within tri-trophic interactions is now well accepted concept. However, communication between different yet closely related plant species and the effect of the distance on their communication remains understudied. To fill in these knowledge gaps, we surveyed predation of artificial caterpillars exposed on MeJA-treated trees (pedunculate and sessile oaks) and conspecifics and heterospecific trees (pedunculate and sessile oaks respectively) standing in their neighbourhood in different distances (2 and 5 m). We found that the artificial caterpillars placed on MeJA-sprayed trees had highest chances of mortality, and the mortality decreased with the distance faster when the treated tree was surrounded by heterospecific trees, than when it was surrounded by conspecific trees. Predation on heterospecific trees at any distance and conspecific trees 5m away from the MeJA-sprayed trees was then significantly lower than on the MeJA-sprayed tree. Natural enemies, and mainly insectivorous birds, seemed to be attracted to MeJA-sprayed saplings of pedunculate and sessile oaks and to their conspecific neighbours standing close to them.

Introduction

Evidence for plant-plant communication accumulating over the years led us from scepticism (Baldwin & Schultz, 1983; Dicke & Bruin, 2001; Rhoades, 1983) to wide acceptance and a description of various aspects of plant communication (Dicke et al., 2003; Pickett et al., 2003). Despite two-way communication via volatiles above-ground and/or through the root system below-ground (e.g. (Dicke & Dijkman, 2001), majority of studies focused on above-ground communication (Dicke et al., 2003).

Natural enemies of herbivores are attracted by the blends of volatile organic compounds (VOCs) emitted by herbivore-infested plants to localize their prey (Turlings & Wäckers, 2004). (Dudareva et al., 2006; Knudsen & Tollsten, 1993; Peñuelas & Llusà, 2004; Steeghs et al., 2004). Undamaged neighbours could benefit from reacting to this information if can recognize VOCs emitted from attacked plants and use them to start their own VOCs signalling for the attraction of natural enemies of herbivores (Landis et al., 2000; Sabelis et al., 1999), or synthesising defensive chemicals against pathogens (Shiojiri et al., 2006) and herbivores (Heil, 2004, 2014).

Some signalling compounds occur widely across plant taxa, whereas others are specific to a plant species or genotype, or the herbivore species triggering their release by feeding on the plant (Douma et al., 2019). This turns the plant communication complex. To localize an herbivore by VOCs, the cue needs to be reliable in time and space. Further, some compounds are emitted quickly upon herbivory and their production quickly ceases when herbivory stops, providing a better signal than the compounds not so closely tied to herbivory.

Most existing studies of plant-plant communication have focused on the responses of conspecifics (e.g. (Dolch & Tschardtke, 2000; Kalske et al., 2019; Karban et al., 2013; Karban et al., 2014). However, interspecific plant communication was also documented (e.g. (Farmer & Ryan, 1990; Glinwood et al., 2004; Karban et al., 2000; Oudejans & Bruin, 1994) for some, but not all, neighbouring species (Peñuelas & Llusà, 2004).

The same species recognition hypothesis assumes that plants react more effectively to the cues emitted by closer than more distant relatives (Karbon et al., 2013). The VOCs are highly variable among species and even individuals, and there is a significant correlation between phylogenetic relatedness and volatile similarity (Karbon & Shiojiri, 2009; Karban et al., 2013). According to the mutual benefit hypothesis, communicating the risk of herbivory benefits the individual emitters regardless of their relatedness to the receivers if the information exchange promotes herbivore movement away from the emitter or patch of neighbours (Bruin & Dicke, 2001; Heil, 2014; Heil & Karban, 2010). Inducing resistance in neighbouring plants may facilitate dispersion of herbivory and reduce the probability of herbivores entering the ever-increasing patch of resistant plants, yielding a benefit to the emitter (Kalske et al., 2019; Rubin et al., 2015).

Distance is another crucial parameter in the plant-plant communication because volatiles diffuse in the air. The distance over which VOC's can affect other plants therefore depends on the sensitivity of these plants to VOCs, as well as the concentration and the lifetime of the emitted compounds (Douma et al., 2019). The information value of the VOCs signal also depends on the foraging behaviour of the herbivores, i.e. how likely they are to spill over to the neighbouring plants. If the herbivores

are sedentary and do not move often from a single plant, or on the other hand tend to bite here and fly far away to bait again, there is no point for the neighbouring trees to respond to VOCs. Many researchers have doubted the ecological relevance of the plant-plant communication, given that positive reports of plant-plant communication were restricted to very short distances (e.g. (Dicke & Bruin, 2001; Fowler & Lawton, 1985; Heil & Karban, 2010; Peñuelas & Llusà, 2004).

The production of VOCs is mediated, at least partly, by the jasmonic acid signalling pathway (Thaler 1999; Thaler et al. 1996), and similar VOCs to those induced by herbivores can often be induced by exposing plants to methyl jasmonate (MeJA) (Dicke, 2015; Dicke et al., 2009). Several studies have shown that application of MeJA on trees or shrubs in nature leads to increased attractivity of such individuals to herbivores' natural enemies (Mäntylä et al., 2014; Schiebe et al., 2012; Zhang et al., 2009) Other studies however did not confirm that application of MeJA attracts predators (Saavedra & Amo, 2018).

In the current study, we test the effects of VOCs after MeJA application on conspecific and heterospecific plants, also examining the effect of distance on plant-plant communication. We use an experimental set-up comprising conspecific and interspecific clusters from pedunculate and sessile oak saplings that had been previously shown to respond to MeJA application (Mrazova et al. in prep). We test the following hypotheses (Fig. 1): 1) Conspecific neighbours of a MeJA-sprayed sapling will attract a higher number of natural enemies than the neighbours of a different species. 2) Neighbouring saplings standing close to the MeJA-sprayed sapling will attract more predators than individuals further away (Fig. 1). 3) Saplings neighbouring to pedunculate oak will attract a lower

number of natural enemies than saplings neighbouring to sessile oak in conspecific clusters (Mrazova et al. in prep).

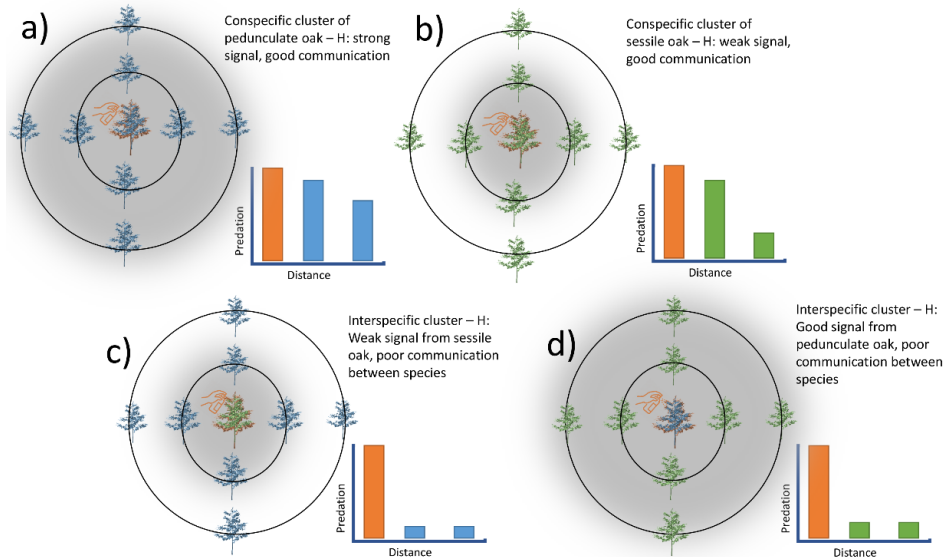


Figure 1. Hypothetical patterns in predation (graphs) under several hypothetical situations where MeJA sprayed pedunculate oak (central saplings, marked by red) provides stronger signal which transfers far away (a, d) than sessile oaks (b, c), and conspecific plants have higher potential to communicate the signal (a, b) than heterospecific plants (c, d).

Methods

We conducted our experiment in a mixed temperate forest close to Dubne village near Ceske Budejovice in Czech Republic in summer 2020. Specifically, the experiment was conducted within a forest gap (GPS location: 48.966817, 14.350394; Fig. S1), surrounded by a mixed-species and structurally diverse forest with tree ages ranging from ca. 10 to 70 years, supporting a high diversity of birds in various vertical strata.

We used two plant species in the experiment - sessile oak (*Quercus petraea*) and pedunculate oak (*Quercus robur*). Both species grow

naturally in the nearby forests but pedunculate oaks are more abundant. The saplings of focal species were purchased in a tree nursery in late February 2019. Saplings were 1.5-2 m tall and planted in 2 l containers. Just after purchase, we transplanted the saplings individually into 80 l containers filled with garden substrate with active humus (AGRO Czech Republic) in early March. First, the saplings were placed outdoors in the campus of the Biology Centre of Czech Academy of Sciences in Ceske Budejovice, where they were watered regularly once a week. After the first flush of leaves on 28th of April 2020, the saplings were translocated to the experimental forest site. We allowed five weeks for the saplings to acclimatize and natural predators to get used to the new tree individuals in buckets in the forest gap. No further watering was needed due to heavy and frequent rains in those weeks. The first experiment testing the simple effect of application of MeJA on our trees was conducted between 20th and 31st May 2020 (Mrazova et al. in prep). The experiment described here was then conducted in June and July 2020.

To investigate the specificity of plant-plant communication and its change due to physical distance between the individuals, we positioned the saplings into clusters where a central sapling was surrounded by eight saplings of the same (conspecific clusters) or different species (interspecific clusters) at distances 2m and 5m from the central treated plant in the four cardinal directions (Fig. S2). In total, we used 72 saplings in the experiment (8 clusters * 9 trees). We allowed at least 20m between the clusters to assure independency of the data. Given the forest gap where the experiment was held is long and narrow, we placed the clusters in two rows close to the forest edge so the birds from surrounding trees would be willing to enter the forest gap to search for potential prey on the saplings.

Experimental setup

Following methods in previous studies, we sprayed the central (MeJA treatment) saplings with 20 ml of 15 mM solution of MeJA solution (consisting of 94.55% of distilled water, 5% ethanol, 0,35% MeJA and 0.1% Tween20 – all from Sigma Aldrich; (Mäntylä et al., 2014)). The first application was made in the early morning hours of June 9 (day 0), and we reapplied the MeJA after 24 hours (experimental day 1 - 10th of June) and then every 48 hours. We run the experiment for 17 days, thus we sprayed the central saplings nine times. All neighbouring trees were sprayed with 20ml of control solution (consisting of 94.9% of distilled water, 5% ethanol, and 0.1% Tween20 – all from Sigma Aldrich; (Mäntylä et al., 2014)) at the times when MeJA was applied.

To study the attractiveness of the saplings to herbivores' natural enemies, we used artificial plasticine caterpillars as an herbivore surrogate (Howe et al., 2009). To prepare artificial caterpillars, we pressed green modelling clay (prepared from 6:1 mixture of light green and brown plasticine, Koh-I-Noor Hardtmuth, Ceske Budejovice, Czech Republic) through a stainless-steel clay extruder gun (Antoble) and made absolutely smooth-surfaced caterpillars (diameter = 3 mm, length = 20 mm). The artificial caterpillars resembled a generic non-conspicuous lepidopteran larva commonly found on oaks in Central Europe (e.g. autumnal moth *Epirrita autumnata*, winter moth *Operophtera brumata*). Between 7 and 8 AM of the first experimental day (day 1), we exposed artificial caterpillars randomly on all focal saplings (neighbouring and MeJA-sprayed) and fastened each of them using an entomological pin (Mrazova & Sam, 2018; Sam et al., 2015). We pinned five dummy caterpillars on the base of leaf stalks in each tree, with a minimal distance of 30 cm from each other. In 48

hour periods, matching the MeJA or control solution applications, we checked the condition of the artificial caterpillars and replaced the damaged ones with new ones pinned on a slightly different location of the same or a neighbouring leaf (Howe et al., 2009). An artificial caterpillar was considered damaged by insectivorous predator if it had some unequivocal bite marks distinctive to predators (Low et al., 2014). At any given time, there were a total of 360 artificial caterpillars exposed to predators. Given all the caterpillars were checked eight times, this yielded 2,880 potential predation events.

Statistical analyses

We employed generalized linear mixed models to assess the effect of the treatment (neighbouring to the sapling of the same or different species, i.e. belonging to conspecific or interspecific cluster respectively), distance for the central sapling (2 or 5 m), and direction from the central MeJA-sprayed tree and oak species on the caterpillar predation rates. The factors above were modelled as fixed effects, with experimental cluster as random effect. As response variable, we used the summed predation rate of caterpillars in different trees along the whole experiment. Response was either total predation, predation by birds or predation by arthropods, in all cases modelled as a binomial variable with a logit link function. For each of these response variables, we employed model selection on a set of biologically relevant candidate models (Table 1), starting with a model including all the variables with additive effects and a subset of meaningful interactions (see full model on Table 1). Models were built using the *glmer* function in the *lme4* package, and all potential models for each response variable were then compared by using the *AICctab* function in package *bbmle*.

Results

We exposed a total of 360 artificial caterpillars which we checked every 48 hours. Over the course of the experiment, this resulted into 5,760 caterpillar-days of exposure, during which we identified 121 (2.1%) attack attempts. The majority of the attacks (85) were caused by birds while 36 attacks were caused by predatory arthropods, which were difficult to identify further (Low et al., 2014). We were not able to find 49 caterpillars (0.8%), which went missing from the saplings during the experiment; they were excluded from the analysis.

The interaction of the cluster type (conspecific or interspecific) and distance of the neighbouring sapling from the central MeJA-sprayed sapling had a significant interactive effect on the total number of attacks on the neighbouring trees (Table 1). Species of the MeJA-sprayed central sapling did not have significant effect on the total number of attacks on neighbouring saplings (Table 1), despite artificial caterpillars on MeJA-sprayed pedunculate oaks suffered slightly higher predation than on sessile oaks ($16.2 \pm 0.6\%$ and $7.1 \pm 0.2\%$ in 48 hours respectively). Specifically, mean total predation on MeJA-sprayed saplings after 48 hours was $11.7 \pm 0.2\%$ (Fig. 2a) and reduced to an average of $6.8 \pm 0.5\%$ at conspecific saplings and $1.3 \pm 0.01\%$ at sapling of different species 2 m away. Five metres from the central MeJA-sprayed saplings, predation was $3 \pm 0.2\%$ on conspecific saplings and $2.5 \pm 0.1\%$ on saplings of different species (Fig. 2a). Caterpillars on pedunculate oaks suffering higher predation than caterpillars on sessile oaks although not significantly (8.3 ± 0.1 vs. $5.3 \pm 0.1\%$ on respective conspecific pairs 2 m apart).

Birds caused the majority of the attacks, and thus the trends in predation by birds were very similar to those observed for total predation

(Table 1; Fig. 2b). In contrast, the very low predation rate from arthropods made all the potential effects non-significant, with the null model performing best. Only treatment was close to showing an effect, but that was not significant (Table 1; Fig. 2c).

Table 1. Model selection based on Akaike criterion.

Model	Total predation		Bird predation		Arthropod predation	
	df	dAICc	df	dAICc	df	dAICc
Treatment*Species+Distance+Direction+Species:Distance	10	13.1	11	15.7	15.7	15.7
Treatment*Species+Distance+Direction	9	10.6	8.8	12.9	12.9	12.9
Treatment*Species+Distance	6	7.1	6.5	5.5	5.5	5.5
Treatment*Distance	5	0	0	3.9	3.9	3.9
Treatment+Distance	4	7.5	5	2.9	2.9	2.9
Treatment*Species	5	8	10.5	3.4	3.4	3.4
Treatment+Species	4	9.9	10.8	2.9	2.9	2.9
Treatment	3	8.4	9	0.8	0.8	0.8
Species	3	18	21.1	1.8	1.8	1.8
Null model	2	15.9	18.9	0	0	0

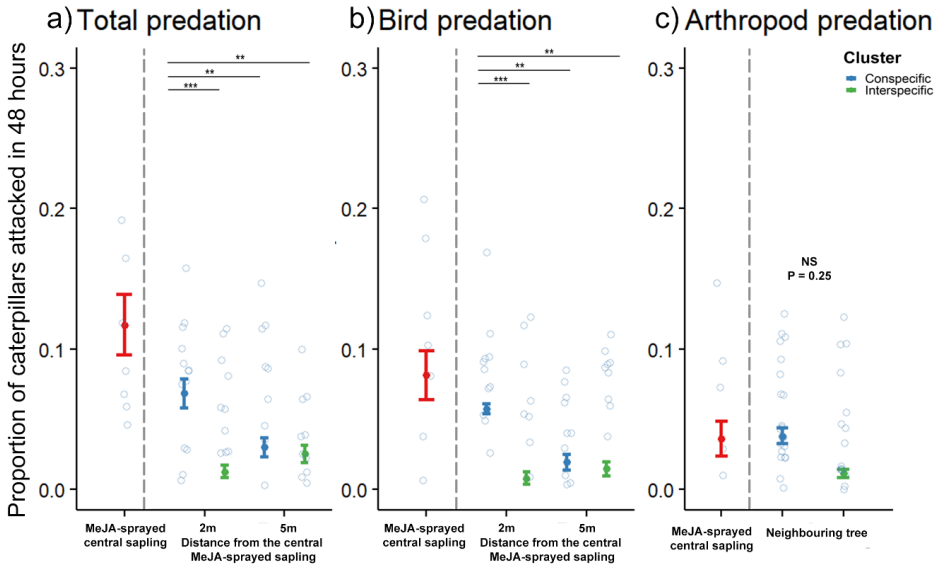


Figure 2. Mean total predation (a), predation by birds (b) and predation by arthropods (c) on central MeJA-sprayed saplings and on the neighbouring saplings in conspecific and interspecific clusters. Saplings of sessile oak (*Quercus petraea*) and pedunculate oak (*Quercus robur*) were used in the experiment, organized into conspecific or heterospecific clusters of nine sapling, where the central sapling was sprayed by methyl jasmonate (MeJA); as the sapling species did not have an effect of predation the results are averaged across species. Statistical differences between the neighbouring saplings are result of emmeans post-hoc test where *** $p < 0.001$ and ** $p < 0.01$.

Discussions

Our results indicate conspecific, but not interspecific, communication between neighbouring oaks by air. We showed in the previous (Mrazova et al. in prep) and this experiment that application of MeJA leads to increased attractivity of natural enemies of herbivorous insect on the sprayed and close-by standing conspecific saplings. Specifically, birds inflicted more than three times higher damage to artificial plasticine caterpillars that were on conspecific oak saplings standing 2m from MeJA-sprayed oak than to

caterpillars on the saplings of different species in the same distance or on at any oak 5 m away from the central MeJA-sprayed sapling. Based on the pattern in our results, we expect that birds were directly attracted to neighbouring saplings that were induced by the MeJA sprayed individuals with the induction depending on their distance from the treated central tree. Both total predation and predation by birds on the central MeJA-sprayed saplings were higher than on any of the neighbouring saplings, although this result was not statistically tested. Predation by arthropods did not differ between the neighbouring trees, and even the predation on MeJA-sprayed trees seemed to be very similar to it.

Mean total predation on MeJA-sprayed oaks was 11.7 ± 0.4 % in 48 hours, which is a rate similar to the one obtained on MeJA-sprayed grey willows at nearby study site ($8.1 \pm 1.3\%$ in 24 hours, (Mrazova & Sam, 2018)). The mean predation on neighbouring saplings 5 m away (2.9 % and 2.5 % in 48 hours) from the central MeJA-sprayed saplings did not seem to differ from predation rates on control saplings in other temperate studies where the mean predations ranged from 1.6% (Drozdová et al., 2013) to 4.3% (Mrazova & Sam, 2018).

As in other similar studies from temperate forests, the birds were responsible for majority of all recorded attacks, while predation by arthropods was usually low (Drozdová et al., 2013; Mrazova & Sam, 2018; Šipoš et al., 2012; Zvereva et al., 2019). Two aspects should be kept in mind, when discussing our results. First, the survey method with plasticine caterpillars is not suitable for absolute measure of predation by different predators and might underestimate attacks of enemies which are not attracted to plasticine (Howe et al., 2009). Second, the saplings were planted in large planters, which could have theoretically prevented access

of some crawling arthropod predators. Using saplings in planters prevented the root communication between plants and allowed for the desired spatial design. During the experiment, we observed some ground ants visiting saplings in large numbers, suggesting that the access by wingless predators was not completely blocked. Although the experimental design could lower the overall predation rates by arthropods, the low predation rates by arthropods were probably not totally unrealistic, which is also suggested by their similarity to predation rates by arthropods in many other temperate studies (Drozdová et al., 2013; Mrazova & Sam, 2018).

Our current data implied and supported thus trend observed in our previous study (Mrazova et al. in prep), that artificial caterpillars exposed on MeJA-sprayed pedunculate oaks were predated more often than caterpillars exposed on MeJA-sprayed sessile oaks. We however did not confirm our subsequent hypothesis, that predation on close by standing saplings will be affected by the identity of the plant species. Both sessile oaks and pedunculate oaks attracted similar number of predators when standing 2 m from MeJA-sprayed sapling conspecific to them.

From previous results (Mrazova et al. in prep; Volf et al., 2020), we knew that exogenous application of MeJA increases production of volatile compounds MeJA on both focal oak species. Both sister species also differ in their chemistry and the inducibility of constitutive defences (Moreira et al., 2018). As our design prevented the communication by root system, allowing only communication by air between the individuals, we assumed the communication via volatile compounds. However, and unfortunately, we did not measure production of volatile compounds in our neighbouring saplings and we have only profiles of VOCs from directly MeJA-sprayed saplings available (Mrazova et al. in prep). Other mechanisms than plant-

plant communication leading to increased production of both saplings in the pair and thus increased predation on both of them might have been happening.

For example, it could be that only MeJA-sprayed saplings actively attracted birds, and the birds coming to them spotted artificial caterpillars on the saplings in their surroundings, without being actively attracted by volatiles of neighbouring trees. Under this scenario, we would likely see no difference in attack rates between conspecific and interspecific clusters, which was not the case in our data. We likely wouldn't see any attractivity of the neighbouring saplings to arthropods. And despite our results are not significant due to the low attack rate by arthropods, the trend of conspecific saplings having similar attack rate as MeJA-sprayed and higher than saplings of different species is detectable in our data.

Another option might be that application of exogenous MeJA itself diffuses in the air and directly affects the nearby saplings. To prevent this possibility, we were spraying MeJA directly and from very short distance on the foliage of the central sapling only, and did not experience any strong winds during the experimental days. Again, it would be unlikely to see the difference in attractivity of conspecific and interspecific pairs of saplings to predators. We therefore believe it is safe to assume that the mechanism behind the data observed by us are resulting from plant-plant communication via volatile compounds in the air.

In previous studies, plant-plant communication by air was described for distances up to 60 cm in sagebrush sprayed by MeJA (Farmer & Ryan 1990, Karban et al. 2006), and but even 15 m (Douma et al., 2019). In other studies, plants separated by 8 m were considered independent, without potential communication via volatile compounds (Dicke et al.

1999). Our data indicate that our saplings communicated only when they were 2 metres apart, and did not communicate when they were 5 m apart. In the view of other existing studies (Douma et al., 2019), we assume that the distance might be relative to the size of experimental plant, and to the amount of MeJA-sprayed or foliage damaged by herbivores. In our study, the saplings were up to 2 m tall, and had ca. 100 leaves. The 20 ml of MeJA was just enough to spray all of them. For example, many VOCs from adult black poplars had highest concentrations between 10 and 15 metres above ground, where majority of its foliage occurred, and ca. 12 metres from the studied trees (Douma et al., 2019).

VOCs can play a prominent role in complex environments such as forests, as they may help natural enemies of insect navigate efficiently through the dense foliage towards their prey (Amo et al., 2013; Turlings & Erb, 2018). The strongest signal might be observed at the site of herbivore attack, weaker signal at the level of whole plant. Additionally, the structure of foliage, canopy and abiotic conditions can modify how the signal spreads further, affecting its efficiency (Douma et al., 2019; Volf et al., 2020). In previous study involving pedunculate oaks, systemic upregulation suggesting transfer by floem within the plant was found for two VOCs however most of the compounds were upregulated in MeJA-sprayed branches locally only (Volf et al., 2020). In particular, terpenoids are reliable infochemicals for predators as they are produced only after herbivore attack and when the leaves are actually damaged (Dicke et al., 1990). Yet, in production of terpenoids in one branch attracted predators which extended their search for prey to surrounding branches or whole canopy (Volf et al., 2020), where the branches were circa 50 cm apart.

In conclusion, natural enemies, and mainly insectivorous birds, seemed to be attracted to MeJA-sprayed saplings of pedunculate and sessile oaks and to their conspecific neighbours standing close to them. However, artificial caterpillars on saplings only 5 metres away from the MeJA-sprayed saplings, and on saplings of different species 2 m away, suffered significantly lower predation than caterpillars on the conspecific saplings 2 m from MeJA-sprayed individuals. The predation on neighbouring saplings were not affected by the species, despite the caterpillars exposed on MeJA sprayed pedunculate oaks suffered higher predation than on sessile oaks.

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Summary

Summary

The aim of the thesis was to describe mechanisms and ecological importance of interactions between plants, herbivorous insect, and predators. Specifically, I focused on role of indirect plant defence in tri-trophic interactions. Further, I focused on communication of plants with higher taxa and its impact on changes in predation pressure. Finally, I studied insect communities in response to defensive chemical compounds released from herbivore-damaged plants.

Main findings

In **Chapter I**, we summarized existing knowledge about birds' use of plant volatile cues for communication within tri-trophic interactions. In accordance with the methodologies used in previous studies, we provided a summary of experimental designs, and we described the advantages and disadvantages of used approaches. We also recommended experimental methodologies that could lead to a deeper understanding of the topic, including considerations of induction of plant defence mechanisms and bird adaptations. Finally, we suggested several interesting questions for future research.

In **Chapter II**, we investigated whether potential differences in the composition of volatile compound profiles produced by Pyrenean oaks (*Quercus pyrenaica*) after herbivorous attack or induction by methyl jasmonate (MeJA) are related to differences in attractiveness to birds, as suggested by the results of Chapter I. The results showed that total volatile emissions produced by MeJA-treated and herbivore-damaged trees did not significantly differ, although MeJA treatments showed a more pronounced response and released several specific compounds in contrast to herbivore-damaged trees.

In **Chapter III**, we examined whether chemical signals from MeJA-treated trees attract insect predators in a complex tropical forest environment and how their attractivity varies with altitude, where predator communities vary naturally. We conducted our research at four study sites along Mount Wilhelm in Papua New Guinea. The main result showed that inducing plant defences by MeJA increased chances of predation of a caterpillar about twice (i.e., plasticine caterpillars exposed on MeJA-treated trees were attacked twice as often as caterpillars exposed on control trees). Arthropod predators (i.e., ants and wasps) caused relatively more attacks in the lowlands (200–700 m asl), while birds became the dominant predators above 1700 m asl.

In **Chapter IV**, we studied how the chemical inducible defence differs between two closely related plant species (English oaks – *Quercus rubra* and sessile oaks – *Quercus petraea*), how it affects the predation on given species and their insect communities. The overall predation was significantly higher on trees treated with MeJA, regardless the oak species. However, studied oak species differed in total emissions of defensive chemical compounds. While the total emissions produced by MeJA-treated sessile oak increased over the time (i.e., over the duration of experiment consisting of multiple applications of MeJA), the amount of compounds emitted by English oak decreased over the time.

In **Chapter V**, we tested whether the Great Tit (*Parus major*) and Blue Tit (*Cyanistes caeruleus*) use chemical (i.e., herbivore-induced volatiles) or visual signals (i.e., herbivore damage) to locate herbivore-rich trees. The results showed that both bird species preferred to search on European privet (*Ligustrum vulgare*) infested with ailanthus silkworm (*Samia Cynthia*)

Summary

providing (1) a combination of visual and chemical signals over a control tree providing no signal, and (2) a chemical signal over a visual signal.

In **Chapter VI**, we surveyed predation of artificial caterpillars exposed on MeJA-treated trees (English oaks and sessile oaks) and on conspecific and heterospecific trees (pedunculate oaks and sessile oaks respectively) standing adjacent to them at different distances (2 and 5 m). We found that artificial caterpillars placed on MeJA-sprayed trees had the highest chance of mortality and that their mortality decreased faster with distance when the treated tree was surrounded by heterospecific trees than when it was surrounded by conspecific trees. Predation on heterospecific trees at any distance and on conspecific trees 5 m away from MeJA-sprayed trees was then significantly lower than on MeJA-sprayed trees.

In summary, the results of this thesis demonstrated that at least some insectivorous birds are able to use olfactory signals to locate insect prey. Insectivorous birds were generally more attracted to plants which produce herbivore induced (or methyl jasmonate) induced plant volatiles, both in the wild in temperate region, in the wild in tropical region as well as in aviaries. Overall, the thesis first summarized the existing knowledge on the topic in a review, and then focused on several specific and understudied topics related to it, providing new insights and methodological recommendations.

Curriculum Vitae

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Employment:

2017 – ongoing: Biology Centre CAS, Institute of Entomology, Associate researcher

2021 – ongoing: Faculty of Science, University of South Bohemia, Consultant, Data manager

2017 – 2020: Biology Centre CAS, Institute of Entomology, Data collection manager (LifeWebs Project)

2017 – 2018: Faculty of Science, University of South Bohemia, Associate researcher

2015 – 2016: CzechGlobe, Global Change Research Institute of the Czech Academy of Sciences, Technician

Education:

2017 – expected defence in August 2022: PhD study at University of South Bohemia, specialization Zoology (Thesis: Interactions between plants, herbivorous insect, and predators: mechanisms and ecological importance, supervisors: Dr. Katerina Sam)


2015 – 2017: Master study at University of South Bohemia, specialization Zoology (Thesis: Can insectivorous birds sense plants calling for help?; supervisor: Dr. Katerina Sam)

2011 – 2015: Bachelor study at University of South Bohemia, specialization Zoology (Thesis: Interspecific interactions of short-toed treecreeper and European treecreeper; supervisor: Dr. Petr Veselý)

Internships:

2017, 2018 (one month each): Prof Manfred Ayasse, Department of Evolutionary Ecology and Conservation Genomics, Ulm, Germany, GC-MS analyses

2018, 2019 (one month each): Dr. Luisa Amo, Department of Evolutionary Ecology, National Museum of Natural Sciences, Madrid, experiments planning and conducting

Publications: Google Scholar: 11 papers, 78 citations, H index 5; **WOS: 11 papers,  47 citations, H index 4** <https://orcid.org/0000-0002-9268-0896> / ResearcherID: R-5253-2018.

- Romero, G., Gonçalves-Souza, T., Roslin, T., Novotny, V., ..., **Mrazova, A.**, ... & Koricheva, J. (2022). Climate variability and aridity modulate the role of leaf shelters for arthropods: a global experiment. *Global Change Biology*, doi: 10.1111/gcb.16150.
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Conferences:

Mrazova, A. (2021). Might birds green an extensive agriculture? *Over the Horizon and for Mutual Acquaintance, Conference of Ph.D. students, University of South Bohemia*. Oral presentation.

Mrazova, A. (2021) Interactions between plants, herbivorous insect, and predators: mechanisms and ecological importance, *Conference of Ph.D. students of the department of Zoology, Faculty of Science, University of South Bohemia*. Oral presentation.

Mrazova, A., Sam K. (2017). The friendship between predators and plants: importance of predatory vertebrates for plant performance. *Ecology across borders, Ghent*. Poster presentation.

Humlová A., Sam K. (2017). Vycítí hmyzožraví ptáci rostliny volající o pomoc? *Zoological days, Brno*. Oral presentation.

Humlová A., Bonny K., Markis P., Sam K. (2016). Tropical birds can smell trees calling for help along an elevational gradient: An experiment with chemically and manually induced herbivory. *Conference of Society for Tropical Ecology*. Oral presentation.

Granted research projects:

Student Grant Agency of the Faculty of Science, University of South Bohemia. 2013. Interspecific interactions of short-toed tree creeper and European tree creeper. (PI; 400 EUR)

Grant Agency of the University of South Bohemia. 2018 -2019. No. 078/2018/P. Importance of visual and chemical signals in tri-trophic systems. (PI; 13,500 EUR)

Awards:

Specialist board award for an excellent work during PhD, Faculty of Science, University of South Bohemia, 2021.

Martina Roeselová Memorial Fellowship for PhD students and postdoctoral researchers caring for preschool children while actively pursuing a career in natural sciences at a university or at a non-university research organization in the Czech Republic, 2021.

Annual award for the best student's ethological publication awarded by Czech and Slovak ethological society 2020; 2nd place.

Teaching, supervising & other scientific experiences:

- Students: University of South Bohemia – 1 Bachelor student defended in 2021
- Regular reviews for 3 scientific journals
- 13 reviews for Student Grant Agency of the Faculty of Science, University of South Bohemia
- Teaching at University of South Bohemia: Field ecology practical course, Field ornithology
- Coordinator of audio recording in LIFEPLAN Global Project: <https://www.helsinki.fi/en/projects/lifeplan> 2020 -2025
- Member of the Ornithology Association of Czech Republic, license no. 1152, 2012 – present
- Popularization lectures for children from the science camp organized by the Faculty of Science, University of South Bohemia

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