Specificity and regulation of chemical defenses in black poplar (*Populus nigra*, L.) against insect herbivores

DISSERTATION

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1 INTRODUCTION

1.1 ANTI-HERBIVORE-DEFENSES IN PLANTS

Plants are sessile organisms and hence unable to flee stress but they are by far not helpless. Beside abiotic stresses like wind, drought, heat, cold and flood, plants must face biotic stresses caused by numerous herbivores and pathogens. Over millions of years, plants have evolved effective systems to deal with environmental conditions and attackers. Herbivory presents an important example of biotic stress and is almost as old as the terrestrial colonization by plants (Labandeira 2007). The long timespan until the present day gave plants as well as their herbivores enough time to develop adaptations toward each other. Plants developed strategies to identify and defend against the attacker while in turn herbivores developed strategies to counteract their recognition or their host's defense in a coevolutionary arms race (Rasmann 2014). Consequently, todays anti-herbivore-defenses in plants as well as their underlying mechanisms are rather complex. Plants fight off herbivores with physical and chemical defenses. Some of these defenses directly target herbivores and are therefore termed direct defenses. Known examples are the vast amount of repellent or toxic compounds occurring in plants. Another type of defense is the recruitment of natural enemies like herbivore parasitoids, termed indirect defense (Paré and Tumlinson 1999; Turlings and Erb 2018). To minimize the disadvantages plant defenses cause, herbivores show behavioral (e.g. trenching, evasion, feeding patterns), physical (e.g. the development of stronger mandibles) or metabolic (e.g. sequestration, detoxification, excretion) adaptations. Additionally they can manipulate the host's phytochemistry for their own benefit (Opitz and Müller 2009; Pasteels et al. 1983). In many cases, herbivores silence or mitigate plant chemical defenses with the help of symbiotic microorganisms (Felton et al. 2014; Giron et al. 2017). It is this complexity that makes the investigation of plant-herbivore-interactions an interesting but also challenging field of research.

1.1.1 Chemical anti-herbivore-defenses

Of all anti-herbivore defenses in plants, chemical defenses are probably the most important factor driving the coevolution of plants and their herbivores (Ehrlich and Raven 1964). Chemical defenses can have repellent and deterrent functions or even cause direct mortality to herbivores (Ibanez et al. 2012). Toxins, growth inhibitors and feeding deterrents are common examples of chemical anti-herbivore defenses synthesized by plants. Additionally volatile organic compounds act intra- and interspecifically

as signals to initiate preparations for an upcoming possible herbivore attack or attract natural enemies of the herbivore. Many chemical defenses are thought to be non-essential for plant growth and reproduction (Wink 2003), but their production is costly. A high investment in chemical defense is accompanied by growth inhibition (Poveda et al. 2003; Zhang and Turner 2008) and lower seed production (Baldwin 1998), although theoretically this cost might be mitigated by metabolic turnover or multifunctionality of such metabolites as well as a broader substrate specifity of enzymes, involved into their biosynthesis (Neilson et al. 2013). However, the limited supply of resources concentrates the accumulation of defense metabolites in the most valuable, fitness relevant parts, as stated by the "optimal defense hypothesis" (McKey (1974). According to this hypothesis, the accumulation of defensive metabolites in different tissues is correlated with the probability of attack, such that tissues subjected to a higher consumption risk are better defended. Reproductive organs and young tissues are often constitutively well defended (constitutive resistance), which means that they generally show increased basal levels of chemical defenses, compared to other, less valuable parts of the plant (Meldau et al. 2012). The costs of constitutive resistance may result in disadvantages in competition with neighboring plants or in the interaction with mutualists under low herbivore pressure, but may pay off during high herbivory pressure, since they are active immediately without a lag in time. However, plants can also increase the concentration of chemical defenses at the onset of herbivore attack, termed induced defense or induced resistance. Induced chemical defenses are widely observed (Baldwin and Ohnmeiss 1993; Piesik et al. 2016; Textor and Gershenzon 2009) and are thought to increase the variability of a plant's defense, which, especially in the light of multiple herbivore species attacking, might be more effective than constitutive resistance (Karban et al. 1997; Zangerl 2003). Which type is more effective might also depend on the plant type.

Investigations about defense responses of plants against herbivores have been primarily carried out with herbaceous plants due to the ease of cultivation, manipulation and carrying out of experiments. However, terrestrial ecosystems are dominated by woody plants with life history traits differing from herbaceous plant species. Trees are perennial plants while herbaceous plants often show annual or biennial life cycles. Their longevity may require different defense strategies. Compared to herbaceous plants the spatial and temporal scale of herbivore attack is much larger. Throughout the season, deciduous trees encounter herbivores from the onset of leaf flushing (Uelmen et al. 2016; van Asch and Visser 2007) until leaf senescence and leaf fall (White 2015). Especially during the growing season, trees are exposed to frequent attacks with individual herbivore species encountered repeatedly. Trees have

much more biomass, however, from which a larger amount of the aboveground organs is concentrated in the stem, making losses of leaves through herbivores less critical. In the years following herbivory events, trees can compensate for tissue losses by enhanced tissue growth or resource reallocation (Edenius et al. 1993; Stevens et al. 2008) although it must be noted that a certain tolerance is observed in herbaceous plants as well (Mabry and Wayne 1997).

1.2 DIRECT ANTI-HERBIVORE DEFENSES

1.2.1 Types of direct anti-herbivore defenses

Anti-herbivore-defenses can directly target the herbivore by preventing it from feeding or inhibiting its growth and development (Howe and Jander 2008). Such direct defenses comprise physical barriers like leaf shape adaptations, trichomes, thorns and spines or morphological features like the fortification of cell walls (Chen 2008; Musariri et al. 2018; Salladay and Ramirez 2019). Physical defenses act as a first barrier against herbivores (Kaur and Kariyat 2020). The second and structurally most diverse type of direct defense is represented by the large group of specialized metabolites, also referred to as secondary metabolites. The term secondary metabolites originated from the hypothesis that these metabolites resulted as by-product of primary metabolism with the high diversity being caused by the play of nature. However, today it is believed that this diversity rather represents an evolutionary adaptation of plants to defend against their attackers (Wink 2003 and references therein). Many of such specialized metabolites have toxic or anti-nutritive effects on herbivores. Modes of action include membrane disruption, inhibition of transport and signal transduction, inhibition of metabolism and the disruption of hormonal control of developmental processes (Gatehouse 2002). The most common groups of specialized metabolites in plants are alkaloids (e.g. cocaine, nicotine), cyanogenic glycosides, terpenoids, glucosinolates and phenolic compounds (e.g. salicinoids, see chapter 1.4.2). Active specialized metabolites are usually accompanied by a variety of related derivative and minor compounds, as observed for terpenoids (Tholl 2015). Additionally to their function as defense metabolites they may have protective function e.g. against UV radiation, serve as nitrogen transport- and storage compounds and take part in pollination (Wink 2003). The third type of direct defense is biochemical- or protein-based defense possessing anti-nutritive and partly toxic functions. This defense limits the food supply prior to ingestion or reduces the nutritional value after ingestion (Chen 2008). Such biochemical defense of plants can affect herbivore growth and development to a great extent. Examples of biochemical defenses are chitinases, proteases, lectines, amino acid deaminases,

polyphenol oxidases and protease inhibitors, which often find their targets in the midgut of herbivores (Napoleao et al. 2019). The most important group, protease and α–amylase inhibitors, inhibit enzymes involved in the breakdown of plant-derived toxic proteins, acting against the herbivore or the digestion of the plant material (Chen 2008). The discovery of protease inhibitors as a defense mechanism of plants was a milestone in the investigation of plant-herbivore-interactions (Green and Ryan 1972). Most plant derived antinutritive or toxic proteins can be easily diminished by the herbivore through proteolysis in its midgut. Protease inhibitors prevent the breakdown of these proteins and prolong their activity. They also reduce nutrient utilization in the herbivore midgut. The inhibition of proteolytic enzymes involved into the breakdown of proteins into amino acids results in a lower availability of essential amino acids crucial for herbivore growth (Mithofer and Boland 2012). The peptidase database MEROPS currently includes 99 families of protease inhibitors distributed over all kingdoms, which shows that they are diverse and widely distributed. Their classification is based on their targeted proteases. The midgut region of the insect herbivore contains four different classes of proteases. Dependent on the pH in the midgut lumen and therefore on the herbivore species, herbivores possess serine- (neutral to alkaline pH), cysteine-, aspartic acid- (acidic pH) and metalloproteases. The most common proteases are the serine proteases, occurring in many herbivores species of the families Coleoptera, Lepidoptera and Orthoptera. Serine proteases can be further subdivided into trypsin-like, chymotrypsin-like and elastase-like proteases (Furstenberg-Hagg et al. 2013). For all of these proteases, plants possess inhibitors, which are often found in plant tissues likely attacked by herbivores, such as apical meristems (Punithavalli and Jebamalaimary 2019), seeds (Samiksha et al. 2019), flowers, rhizomes and roots (Chan et al. 2017). Additionally, protease inhibitors are induced after herbivore attack (Castano-Duque et al. 2018; Chan et al. 2017; Green and Ryan 1972).

1.2.2 Intraspecific variability of direct plant defenses

Given the limited number of biotic and abiotic environmental stresses plants must face, but the enormous number of defensive chemicals described so far, it is clear that the defense response of plants differs between plant species. The best example comes from the group of specialized defense metabolites. For example, while plant species of the Solanaceae family like tobacco and tomato produce alkaloids for anti-herbivore defense (Chowanski et al. 2016) species of the Salicaceae family like poplar and willow trees mainly defend with phenolic compounds (Boeckler et al. 2011). Species of the Brassicaceae family like broccoli often defend themselves using the glucosinolates-myrosinase system (Halkier and Gershenzon 2006) while conifers like spruce (*Picea* spp.) and pine (*Pinus* spp.) trees rely

upon terpenoids to defend against herbivores (Celedon and Bohlmann 2019). Within single species chemical defenses vary between genotypes leading to differences in herbivore susceptibility, which can influence plant- and herbivore development and thus shape herbivore communities (Barker et al. 2019; Bravo-Monzon et al. 2014; Kleine and Muller 2011). Intraspecifically, the genotype is probably the most influential factor determining the defense responses of plants. In addition to the genetic variation among plants, a single individual also varies in its anti-herbivore defenses. The chemistry of a plant is not homogenous but rather fluctuates depending on organ and stage of development. As nicely hypothesized by Whitham (1981) the different distribution of phytochemicals within a plant leads to a mosaic of varying susceptibilities to herbivores and parasites. The adaptation of herbivores to such conditions can be more difficult compared to a homogenous distribution of phytochemicals. This is because herbivores that are not able to discriminate between phytochemical variations either make inappropriate feeding choices, leading to reduced fitness, or otherwise clump at optimal positions, which increases visibility to natural enemies. For herbivores feeding on leaf foliage, seasonal variations in the leaf phytochemistry are a very important predictor of herbivore distribution (Raupp and Denno 1983). Juvenile leaves are full of nutrients essential for herbivores, such as proteins, sugars, water and nitrogen. As leaves mature these nutrients decline and instead fiber content and leaf toughness increase. Lesser nutrient availability paired with a decreased efficiency of nutrient extraction, digestion and absorption negatively impact the development of herbivores (Barbehenn et al. 2015; Hunter and Lechowicz 1992). These factors alone would make the choice of where to feed easy. However, plants often defend juvenile leaf tissues better than mature tissues (Meldau et al. 2012). Herbivores must therefore evaluate the optimal spot for feeding, maximizing nutrient quality and minimizing defenses.

1.3 HERBIVORE PERCEPTION, DEFENSE SIGNALING AND SPECIFICITY OF ANTI-HERBIVORE DEFENSES

1.3.1 Herbivore perception

The successful response of a plant to an herbivore must be quick and accurate. The accuracy of a defense response requires a reliable identification of the attacking herbivore, which is the first and most crucial step in anti-herbivore-defenses. Plants do not possess sensory organs like e.g. antennae, eyes or ears. Instead they have developed other systems for recognition. One of the first steps of herbivore recognition already occurs when the herbivore walks on the plant, breaking trichomes or causing minor scratches on the leaf surface. While feeding on the plant, herbivores leave chemical and mechanical

cues (Hilker and Meiners 2010). Mechanical damage alone can trigger defense responses, but plants can further distinguish it from herbivory by the perception of specific herbivory-associated elicitors (HAEs). Such elicitors were evidenced in herbivore oral secretions (Halitschke et al. 2001), glandular secretions used in oviposition (Voirol et al. 2020), and even frass (Ray et al. 2015). Examples of herbivore-derived elicitors are fatty acid-amino acid conjugates (Alborn et al. 1997), sulfur-containing fatty acids termed caeliferins (Alborn et al. 2007), peptides (Schmelz et al. 2006) or enzymes like ß-glucosidases (Mattiacci et al. 1995) and lipases (Schaefer et al. 2011). Recently, phytohormones present in the saliva of herbivores were reported to modulate anti-herbivore-responses in plants (Acevedo et al. 2019). The perception of such herbivore-associated cues activates signaling cascades resulting in the production of defense metabolites. The simultaneous perception of damage-associated molecular patterns (DAMPs) and herbivore-associated molecular patterns (HAMPs) often amplifies plant defense responses (Halitschke et al. 2001; Schaefer et al. 2011). These signaling cascades then lead to herbivore-triggered immunity (HTI).

1.3.2 Phytohormones and transcriptional regulation of defense responses

Within seconds to minutes, changes in plant membrane potential caused by herbivory activate calcium ion (Ca^{2+}) signaling, followed by the production of reactive oxygen species and activation of MAPKs. MAPKs further regulate the elicitation of plant defense hormones and transcriptional changes involved in anti-herbivore defenses (Furstenberg-Hagg et al. 2013; Hettenhausen et al. 2015). The most important phytohormones known to be involved in the anti-herbivore defense responses in plants are jasmonic acid (JA) and especially its bioactive form the isoleucine conjugate jasmonoyl-isoleucine (JA-Ile), salicylic acid (SA) and ethylene (as modulator of JA and SA regulation). However, because of the complex network of phytohormone interactions, other hormones like abscisic acid, gibberellins, auxins, cytokinins and brassinosteroids also influence defense responses (Meldau et al. 2012). Numerous studies show that the jasmonate pathway plays a central role in plant resistance against biting-chewing insect herbivores and necrotrophic pathogens while the salicylate pathway is more important for resistance against biotrophic pathogens (Acevedo et al. 2015; Glazebrook 2005). Additionally, JA and SA are both involved in plant defense against insect eggs (Hilker and Fatouros 2015). The JA pathway orchestrates the transcriptional regulation of many genes involved in direct and indirect anti-herbivore defense, growth and nutrient signaling as well as reproduction (Armengaud et al. 2004; De Vos et al. 2005; Devoto and Turner 2005; Thaler 1999). Examples of known JA-responsive genes involved in anti-herbivore defenses and anti-egg defenses are genes encoding protease inhibitors and terpene synthases

(Clavijo McCormick et al. 2014b; Farmer and Ryan 1990; Kim et al. 2012; Kopke et al. 2008). Additionally, JA responsive genes regulate the biosynthesis of JA itself as well as the biosynthesis of other phytohormones (Sasaki et al. 2001). The SA pathway is usually activated upon recognition of pathogen-associated molecular patterns (PAMPs) and finally leads to pathogen-triggered immunity (PTI) and effector-triggered immunity (ETI) (Filgueiras et al. 2019). Both immunities can result in apoptosis at the site of infection, a phenomenon termed the hypersensitive response (Heath 2000). Additionally SA is a necessary component of systemic acquired resistance and a multitude of other physiological effects like stomatal dynamics, plant thermogenesis, seed germination, cell growth, vegetative growth, flowering, photosynthesis and responses to abiotic stresses (Filgueiras et al. 2019). Not much is known yet about the transcriptional regulation of SA-responsive genes involved in insect egg defense but results of emerging studies suggest that PR genes might also play a role in this type of defense (Gouhier-Darimont et al. 2019; Lortzing et al. 2019).

1.3.3 Systemic and volatile-mediated signaling

Since phytohormones are able to travel through the vascular system of plants, defenses can be increased in undamaged areas surrounding the attacked site. But other components involved in the systemic induction of herbivore resistance have also been identified. These include systemin peptides found in Solanaceae, other peptides with similar function outside Solanaceae and oligogalacturonides (Furstenberg-Hagg et al. 2013). However, while vascular signaling between leaves is important at close or medium ranges, long distance signaling is realized via a different mechanism. Plants constitutively release volatile organic compounds (VOCs) of which some play important roles in attracting pollinators or seed dispersers. Others are produced or increased specifically after herbivore attack (herbivore-induced plant volatiles – HIPVs) and can attract natural enemies of the herbivore (Clavijo McCormick et al. 2014b; Heil and Silva Bueno 2007). Beside this, they can also act as direct defenses against abiotic and biotic stress. Studies have shown repellent effects of VOCs on herbivores, including females searching for oviposition spots (Bernasconi et al. 1998; De Moraes et al. 2001; Rostas and Hilker 2002). In turn, herbivores can exploit the release of VOCs to find their host plant. Some VOCs possess antimicrobial activity (Quintana-Rodriguez et al. 2018). The complexity of the volatile bouquet as well as the vast amount of volatiles released by plants complicates investigations about their involvement in plant-herbivore interactions. Their release occurs through membranes of the epidermal tissues, trichomes, osmophores, crenulated epidermal cells and through the stomata (Baldwin 2010). Depending on their synthesis the majority of plant volatiles can be classified in the following groups: terpenes or

terpenoids, fatty acid derivatives (green leaf volatiles - GLVs), amino acid derivatives (nitrogen- and sulfur containing volatiles) and aromatic compounds (Baldwin 2010). Synthesis of volatiles is often regulated by jasmonates (Boland et al. 1995; Luck et al. 2016; Semiz et al. 2012) and ethylene (Broekgaarden et al. 2015; Schmelz et al. 2003). The perception of HIPVs by neighboring plants or undamaged parts of the same plant can lead to induced defenses as a preparation for a likely upcoming attack (Heil and Karban 2010). In a slightly different scenario, plans respond to volatile by going into an alarmed state, a phenomenon termed priming. Once the actual attack occurs the defense response of primed plants can be faster and stronger, compared to non-primed plants (Heil and Kost 2006; Kost and Heil 2006). Although the priming mechanism in plants is not fully elucidated today, it is known to involve physiological, metabolic, transcriptional and epigenetic changes (Mauch-Mani et al. 2017). While the investment in priming is thought to be relatively low if the plant is not attacked, models suggest that the fitness benefits can be significantly increased once an herbivore attacks (Douma et al. 2017). Although the mechanisms of priming are largely unknown, the negative influence of priming on herbivores after their attack is evident (Kessler et al. 2006; Morrell and Kessler 2017; Yoneya et al. 2014).

1.3.4 Specificity of plant defense responses

The induction of inducible plant defenses might be specifically tailored to the attacking herbivore species. This is recognized in the literature through terms such as "*specificity of plant responses*" (Karban and Baldwin 1997), "*specificity of elicitation* (Stout et al. 1998) and "*specificity of induced resistance*" (Agrawal 2000), which raise the question of whether plant damage by different herbivore species causes the same or distinct defense responses. One can also ask whether the same plant defense differentially affects herbivore species or their developmental stages (Agrawal 2000; Karban and Baldwin 1997; Stout et al. 1998). Specificity of induced resistance has been detected for many chemical compound classes involved in anti-herbivore defenses, including phytohormones (Agrawal et al. 2014; Kroes et al. 2016), specialized metabolites (Bidart-Bouzat and Kliebenstein 2011; Van Zandt and Agrawal 2004), protease inhibitors (Chung and Felton 2011) and volatile organic compounds (Silva et al. 2017; Unsicker et al. 2015). Specificity of induced resistance can also be measured at the molecular level (Kroes et al. 2016; Stout et al. 1998), especially because it is not always clearly visible at the metabolite level (Chung and Felton 2011). Last but not least specificity of induced resistance can be measured indirectly by plant performance parameters like reproduction and seed production (Moreira et al. 2015; Rusman et al. 2019). While specialized defense responses were observed in many studies, less information on their cause is available. Recent studies showed that herbivore saliva plays an important role (Diezel et al.

2009; Voelckel and Baldwin 2004) but also damage patterns (van Poecke et al. 2003), the level of herbivore specialization (Agrawal 2000; Rowen and Kaplan 2016), the affiliation of herbivores to certain feeding guilds (Agrawal and Sherriffs 2001; Heidel and Baldwin 2004), and the influence of microbes (Acevedo et al. 2015) have been discussed. One of the most prominent arguments so far is the classification of herbivores into feeding guilds. Such a classification distinguishes between piercing-sucking herbivores like aphids or whiteflies and biting-chewing herbivores like caterpillars or leaf beetles. Studies have investigated the feeding-guild dependent specificity of induced resistance and found differential induction patterns of phytohormones, specialized metabolites and VOCs as well as transcriptional differences. In general chewers seem to induce more JA-responsive genes while phloem feeders induce more SA responsive genes (Ali and Agrawal 2012 and references therein). A possible reason might be that phloem feeders cause less tissue damage compared to chewing insects. However, even within a feeding guild plants can respond in an herbivore-species-specific way. For example, Diezel et al. (2009) found differential phytohormonal responses of coyote tobacco *Nicotiana attenuata* when exposed to either oral secretions of the tobacco hornworm *Manduca sexta* or the beet armyworm *Spodoptera exigua*. Other studies also showed such differences especially when comparing the response to moth larvae and leaf beetles (Agrawal et al. 2014; Chung and Felton 2011; Unsicker et al. 2015; Van Zandt and Agrawal 2004). However, this suggests that it is not the feeding guild alone determining specificity of induced resistance. Another intensively discussed argument is whether the level of herbivore specialization affects specificity, but the results of studies addressing this question are highly controversial. While some studies found differential plant responses to specialist versus generalist herbivores other studies could not confirm these results (Ali and Agrawal 2012 and references therein). The inconsistencies arise from methodological challenges in the experiments. First, many studies mix herbivore specialization level and feeding guild. Investigating the specificity of defense responses in plants requires a special design, which includes a sufficient set of herbivore species, optimally with replicated feeding guild and specialization level. However, such studies are scarce. The few examples known could rule out the sole influence of the herbivore specialization level (Agrawal 2000; Bidart-Bouzat and Kliebenstein 2011; Mewis et al. 2006). Second, plant species and genotype, respectively, influence the specificity of induced resistance (Agrawal et al. 2014; Poelman et al. 2008), reducing the comparability to other studies. Third, the level of herbivore specialization cannot be seen as a dichotomy between highly polyphagous and strongly monophagous herbivores. The herbivore specialization level is shaped by many gradations, which might affect the orchestration of plant defense responses. Fourth, herbivores are able to manipulate the plant, influencing its defense response and

therefore challenging investigations about the specificity of induced resistance (Ali and Agrawal 2012). Last but not least, the defense response of plants to their herbivores depends on the herbivore developmental stage. This is especially true for the egg stage. Plants exposed to insect eggs often show completely different responses than to other life stages. Such responses include leaf necrosis, the formation of neoplasm or egg-crushing tissue or the production of ovicidal substances. Plant responses to insect eggs are therefore more similar to the responses to pathogens than the responses to herbivores. The induction of ovicidal substances and herbivore-induced plant volatiles (HIPVs) are known to be herbivore-species specific (Hilker and Fatouros 2015; Hilker and Meiners 2010), which may arise from specific elicitors occurring in ovipositional secretions. Elicitors described include bruchins (Doss et al. 2000), benzyl cyanide (Fatouros et al. 2008), indole (Fatouros et al. 2009) as well as proteinaceous elicitors (Hilker et al. 2005) and phospholipids (Yang et al. 2014). Also microbes have been discussed to be involved in responses of plants to oviposition (Bertea et al. 2020).

1.4 STUDY SYSTEM

1.4.1 Study organisms

In this thesis, the specificity of induced anti-herbivore resistance was investigated using black poplar (*Populus nigra*) trees as a study organism. Lepidopteran herbivores used include the gypsy moth (*Lymantria dispar*), the poplar hawk moth (*Laothoe populi*) and the moth species *Amata mogadorensis*. Leaf beetles used in these studies included the blue willow leaf beetle (*Phratora vulgatissima*) and the poplar leaf beetle (*Chrysomela populi*).

Black poplar is one of about 35 described plant species belonging to the *Populus* genus (commonly referred to as aspen, cottonwood and poplar), which is in the Salicaceae family. It is a deciduous tree species native to Europe, southwest and central Asia as well as northwest Africa. Mature black poplar trees can reach up to 35 m height and 2 m trunk diameter although exceptions can grow even larger. Usually, this tree species reaches an age of 100-150 years. Black poplar trees are dioecious and wind pollinated. They can also regrow vegetatively from stem- or root fragments. Sexual reproduction enhances genetic diversity and is important for the colonization of new habitats while vegetative reproduction is important for the expansion of the population and the creation of structural diversity (Tinschert et al. 2020). As an important pioneer species shaping riparian river systems, black poplar is tolerant to regular flooding and shows rapid growth. As other species closely associated with riparian

river systems, black poplar is an endangered species. The main reason for this is the continuous loss of floodplain river habitats caused by human land use change and agricultural intensification (Krause et al. 2011). Although black poplar naturally shows a substantial degree of genetic variation (Guet et al. 2015), destruction and fragmentation of suitable habitats, intensive monoclonal cultural practices in short-rotation coppices and hybridization events have also caused a reduction in the genetic diversity of this tree species (Ciftci et al. 2017; Ciftci and Kaya 2019). Ecologically, black poplar trees are important as a food source, shelter and habitat for numerous associated organisms. Its ability to grow from stem and root cuttings and its fast growth makes black poplar an interesting model organism to study plant-herbivore interactions in woody plants. Additionally, the genome of the closely related black cottonwood (*Populus trichocarpa)* was already sequenced and published (Tuskan et al. 2006), offering the possibility to investigate molecular biological features like the transcription of genes involved in poplar defense processes. This knowledge improves our understanding of the complex regulatory network behind the induction of defense metabolites.

1.4.2 Chemical defenses in poplar

Poplar trees use a set of direct and indirect anti-herbivore defenses, including phenol-based specialized metabolites, biochemical defenses and VOCs. Phenol-based metabolites like salicinoids, tannins, phenolic acids and flavonols are commonly found in poplar, and can accumulate in significant concentrations. While some metabolites possess anti-herbivore activity, others serve different ecological functions. The flavonoid catechin, for example, was recently shown to be involved into anti-fungal defenses in black poplar (Ullah et al. 2017). Additionally, studies with artificial substrates have shown oviposition-inducing activity of catechin although studies *in situ* remain to be carried out (Islam et al. 1997; Ueno et al. 1990). The role of phenolic acids in poplar remains to be investigated, but studies have shown that some of them like cinnamic acid are important precursors for salicinoids (Babst et al. 2010) or possess antimicrobial activity (Nassima et al. 2019). However, the two major classes of phenolics occurring in poplar are salicinoids and condensed tannins, which can reach 20 % and more of the foliage dry weight (Boeckler et al. 2011; Donaldson et al. 2006). Although both metabolite groups were earlier believed to be effective defense compounds against insect herbivores, only salicinoids still remain to be considered as such. Condensed tannins are now thought to participate less in anti-insect-herbivore defenses but more in defense against herbivorous mammals (reviewed in Barbehenn and Constabel 2011). Salicinoids are the most prominent example of phenol-based direct defenses against insect herbivores (Barbehenn and Constabel 2011; Boeckler et al. 2011). Formerly termed as “phenolic

glucosides", they were renamed because of their exclusive occurrence in Salicaceae plants and their structures, which all contain salicin as a core. Salicinoids are derived from the phenylpropanoid pathway and are built of salicyl alcohol linked to β-D-glucopyranose via an ether linkage between the phenolic hydroxyl group and the anomeric carbon atom of the glucose. Hence, the simplest salicinoid is salicin itself. More complex salicinoids like salicortin and homaloside D additionally possess certain functional groups on the salicyl alcohol or the glucose moiety of the salicin core (Boeckler et al. 2011). Salicinoids are highly abundant in poplar trees and concentrations of up to 30 % of plant dry weight were reported (Donaldson et al. 2006). They show a high genotypic variation (Osier and Lindroth 2001; Osier and Lindroth 2006). Additionally, the season, the plant ontogeny as well as nutrient and water availability influence salicinoid concentrations (Donaldson and Lindroth 2007; Hale et al. 2005; Osier and Lindroth 2006). Anti-herbivore functions of salicinoids were already evidenced for generalist- (Hemming and Lindroth 1995) as well as specialist herbivores (Kelly and Curry 1991) although some specialist herbivore species are less affected and are even attracted to them (Rank 1994; Roininen et al. 1999). However, great uncertainties occur in their inducibility. While some studies showed increased salicinoid concentrations after herbivore perception (Clausen et al. 1989), others failed to confirm these patterns (Boeckler et al. 2013; Osier and Lindroth 2001). However, in the latter two studies all measured salicinoids were analyzed and presented in a summed form but labeling experiments by Babst et al. (2010) indicated that salicin might have a different biochemical background than the complex salicinoids, raising the possibility of a different ecological function.

Beside salicinoids poplar trees also defend themselves with protease inhibitors (Haruta et al. 2001; Major and Constabel 2008; Miranda et al. 2004). One of the most intensively investigated protease inhibitor subfamilies occurring in poplar trees is the Kunitz-type trypsin protease inhibitor (KTI) family. KTIs are relatively small proteins with a size of 20-25 kDa with a ß-trefoil fold consisting of several ß-strands connected by long loops of which one possess the inhibitory activity. Additionally, they usually contain two disulfide bridges formed by cysteine residues of which the one closest to the N-terminus is highly conserved and probably important for the activity (Philippe et al. 2009). The anti-herbivore activity of KTIs has been demonstrated *in vitro* (Garcia et al. 2004; Major and Constabel 2008) and indirectly via performance measurements of the herbivores (Arnaiz et al. 2018; Jamal et al. 2015). Like other protease inhibitors KTIs have been shown to be inducible in response to mechanical wounding and herbivory (Ma et al. 2011; Major and Constabel 2008; Philippe et al. 2009).

As indirect defenses, poplar trees emit a complex mixture of VOCs, partially induced after herbivory. VOCs occurring in black poplar trees can be classified in six large groups. These groups are homoterpenoids, monoterpenoids, sesquiterpenoids, aromatic volatiles, nitrogenous volatiles and GLVs (Clavijo McCormick et al. 2014b). Typical examples of abundant herbivore-inducible plant volatiles belonging to these groups are the monoterpenes linalool and *(E)-ß*-ocimene, the sesquiterpenes *(E)-ß*-caryophyllene and (E,E)-*α*-farnesene, the green leaf volatiles *(Z)*-3-hexenyl-acetate and *(Z)*-3-hexenal, the nitrogenous volatiles 2-methylbutyraldoxime and benzyl cyanide and the aromatic compound indole (for review see Clavijo McCormick et al. 2012). A previous study in black poplar showed that the constitutive volatile emission fluctuates in diurnal patterns. VOCs belonging to terpenes, for example, increase in their concentration during daylight and decline at night, while nitrogenous, aromatic and green leaf volatiles are less light dependent (Clavijo McCormick et al. 2014a). The time-point of herbivore attack thus influences the emission response of certain VOCs, which further affects the recognition of the herbivore by natural enemies. The recognition of herbivore presence by natural enemies depends on the compounds they orient to, which might be a mixture or single compounds. Electroantennogramms showed that nitrogenous volatiles play a predominant role in attracting parasitic wasps to black poplar, but responses to GLVs and terpenes were also observed (Clavijo McCormick et al. 2014b), supporting the theory that herbivores respond to mixtures of volatile compounds. Nitrogenous compounds might be of special importance since they are produced exclusively in herbivore-damaged leaves, rendering them suitable cues for parasites or predators to locate their hosts or prey. Apart from nitrogenous volatiles the majority of herbivore-triggered changes in black poplar volatiles are quantitative (Unsicker et al. 2015). Specificity of the VOC response to herbivores therefore mostly originates from changes in the concentration of the same compounds rather than qualitative differences in the volatile bouquet. Herbivore-species-specific changes in black poplar's volatile emission were observed before, especially when comparing emissions after herbivory by moths and leaf beetles (Clavijo McCormick et al. 2014b; Unsicker et al. 2015) but interestingly not when different moth species were compared. Differences were also observed for different developmental stages of the same herbivore species (Clavijo McCormick et al. 2014a). The specificity of black poplars VOC response to different herbivore species, especially moths and leaf beetles, might also influence the priming effects of attacked plants on neighboring plants and distant parts of the emitter plant. Priming effects within a single host tree have been suggested as a superior way to quickly communicate between distant parts of a poplar host tree, compared to vascular signal transmission (Frost et al. 2007; Li and Blande 2017). Additionally studies in poplar trees could show priming effects on neighboring plants (Frost et al. 2008;

Li et al. 2012). Studies about specific compounds involved in priming on poplar trees are scarce. One of these studies suggested the involvement of GLVs (Frost et al. 2008). However, studies on other plant species have also suggested the involvement of terpenes as signal transporters (Arimura et al. 2000).

1.4.3 Herbivores used in the studies

Unlike most herbaceous plants, black poplar trees are accompanied by numerous herbivores, throughout the whole season. More than 300 insect and mite species have been recorded on 12 poplar species in North America while in Europe there are more than 500 species (Mattson et al. 2001). The most investigated generalist herbivore occurring on poplar is the gypsy moth (*Lymantria dispar*). While this herbivore species is native in Europe, it is a more serious pest in North America. Since its accidental introduction in 1869 in Massachusetts, the gypsy moth has spread and causes regular outbreaks that can defoliate whole forests causing massive ecological and economical damage (Maccini et al. 2018). The gypsy moth is a generalist herbivore feeding on more than 500 coniferous and deciduous tree species, which presents one reason for its success. Adult females of the European gypsy moth (*Lymantria dispar dispar* race *Europe*) are incapable of flight and oviposit egg clutches containing up to 1000 eggs unselectively on bark, tree trunks and branches but also on rocks, outdoor furniture, buildings and vehicles. The young larval stages of this species can be distributed by wind dispersion using silk treads (termed “ballooning”). The gypsy moth usually is univoltine, producing only one generation per year. However, under favorable conditions a second generation is possible (Global Invasive Species Database 2020). Another generalist herbivore used in this dissertation is the moth species *Amata mogadorensis*, a generalist herbivore distributed mainly in Algeria and Morocco. It was recorded feeding on plants of the genera *Sonchus*, *Plantago*, *Vitis* and *Populus* (Savela 2019). One of the most prominent specialist herbivores commonly occurring on poplar trees is the poplar leaf beetle (*Chrysomela populi*). *C. populi* is a widespread beetle species, which can be found in most parts of the Palearctic realm and in parts of the Oriental realm. It has specialized on trees of the Salicaceae family with a preference for willow (*Salix*) and poplar trees, with poplar trees being especially attacked. This multivoltine beetle species usually occurs from April to October and can reach up to 3 generations per year. Larvae overwinter in the litter beneath leaves. Its appearance in spring usually coincides with leaf flushing. It prefers especially young leaves of poplar shoots because they contain high levels of nutrients and salicinoids (Urban 2006). Young larvae use one of the salicinoids, salicin, as precursor to form salicylaldehyde, which is then transported and stored in specialized glands, where it is used as defense compound against predators (Pasteels et al. 1983). The damage pattern of adult *C. populi* is similar to

lepidopteran herbivores while the larvae skeletonize leaves. The preference for young trees and shoots combined with its multivoltinity makes *C. populi* a dangerous threat especially in short-rotation coppices where young poplars are densely planted in monoculture (Schroeder and Fladung 2018). Further important herbivores on black poplar are the specialist poplar hawk-moth *Laothoe populi* and the specialist blue willow leaf beetle *Phratora vulgatissima*. The nocturnal poplar hawk-moth is found throughout the Palearctic realm and the Near East. It feeds on Salicaceae plants with a preference for poplar and aspen trees but depending on the location willows are also potential food sources. The poplar hawk-moth usually is univoltine but can have a second generation under advantageous circumstances. This species occur from May till July and usually prefers mature black poplar leaves over young shoots (Williams 1966). The blue willow leaf beetle (*Phratora vulgatissima*) is another specialist herbivore on Salicaceae trees feeding on poplar trees but it prefers willows, where it can cause severe damage in plantations (Stenberg et al. 2010). Although this species is univoltine it can have more than one generation per season if conditions are favorable. It overwinters as adults beneath tree bark or lichens (Kendall and Wiltshire 1998). Populations occur from April till August/September. The feeding preference of the blue willow beetle is inversely related to the concentration of salicinoids, and especially the concentration of the complex salicinoid salicortin (Kelly and Curry 1991). However, it prefers to feed on shoots, where it chews holes and notches into the leaves, leaving them skeletonized.

1.5 AIM OF THE THESIS

Rigorous investigations about the specificity of plant defenses against herbivores are not easy to perform. As mentioned above, many studies addressing this question used too few herbivore species to draw firm conclusions or mixed feeding guilds and level of specialization during their experiments. Additionally most of the studies investigating specificity of the induced resistance were conducted on herbaceous plants, while woody plants are clearly underrepresented. This thesis therefore concentrates on the specificity of black poplar defense responses to herbivores from a single feeding guild, namely the guild of biting-chewing herbivores. Another aim of this thesis was to address the specificity of poplar defense responses on generalist and specialist herbivore species. Some behavioral adaptations of herbivores to such defenses were investigated, such as the feeding and oviposition strategies of *C. populi*. A special focus lies in the involvement of direct defenses like salicinoids and protease inhibitors in shaping these interactions, but also indirect defenses were investigated.

2 OVERVIEW OF THE MANUSCRIPTS

2.1 CHAPTER I: SPECIFICITY OF THE CHEMICAL DEFENSE IN BLACK POPLAR

Specificity of Herbivore Defense Responses in a Woody Plant, Black Poplar (*Populus nigra*)

Thomas Fabisch, Jonathan Gershenzon & Sybille B. Unsicker

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During this study we investigated the specificity of black poplar defense response to different insect herbivores belonging to the same feeding guild. Herbivores used in the experiments of this study were the two generalist moth species *Amata mogadorensis* and *Lymantria dispar*, the specialist moth *Laothoe populi* as well as the two specialized chrysomelid species *Phratora vulgatissima* and *Chrysomela populi*. Herbivory triggered changes in phytohormones and a Kunitz-type trypsin protease inhibitor, but showed little tendency towards herbivore-specific differences. Salicinoids, a major anti-herbivore defense of Salicaceae against insect herbivores, were not affected by herbivory except salicin, which showed no herbivore-specific patterns. In contrast, volatiles responded in herbivore-specific fashion, especially when comparing lepidopteran with coleopteran herbivore species. This study shows that the majority of direct defenses in black poplar are not herbivore-species specific, but differences in induction patterns can be found for indirect defense compounds.

2.2 CHAPTER II: MOLECULAR MECHANISMS BEHIND BLACK POPLAR DEFENSES

Poplar protease inhibitor expression differs in a herbivore species-specific manner

Franziska Eberl, Thomas Fabisch, Katrin Luck, Tobias G. Köllner, Heiko Vogel, Jonathan Gershenzon & Sybille B. Unsicker

Published *in BMC Plant Biology,* April 2021, Volume 21, Issue 1, Article 170, p 1-11

This study focused on the identification and transcriptional regulation of black poplar genes encoding Kunitz-type trypsin protease inhibitors (KTIs) in response to the attack by two lepidopteran herbivore species, *Amata mogadorensis* and *Lymantria dispar*, as well as the coleopteran herbivore species *Phratora vulgatissima*. Seventeen KTI genes were identified and sequenced and eight shown to be up-regulated in response to herbivory via qRT-PCR. Beetle herbivory elicited stronger transcript expressions compared to the two lepidopterans, which were similar in their intensity. The transcriptional patterns of KTI genes correlated with trypsin-inhibitor activity tested in herbivore-damaged leaves, although they were not dependent on leaf area loss, suggesting a threshold-based induction. With this study we show that black poplar protease inhibitors are induced in a herbivore -specific fashion and are controlled at the transcriptional level.

2.3 CHAPTER III: VOLATILE PRIMING IN BLACK POPLAR

Volatile mediated defense priming in black poplar. Minor changes can cause major differences

Sandra Lackner, Thomas Fabisch, Heiko Vogel, Beate Rothe, Jonathan Gershenzon & Sybille B. Unsicker

In preparation for *Ecology Letters*

This study aimed to investigate herbivory-triggered volatile mediated priming in young black poplar trees. Receiver trees were exposed to the volatile bouquet of emitter trees, either those that experience herbivory by gypsy moth caterpillars or undamaged controls. Although the volatile bouquet of the herbivore-infested emitters was quantitatively different from the non-infested controls, the phytochemistry of the receiver trees was unchanged. However, after subsequent exposure of the receiver trees to gypsy moth herbivory, the induction of the salicinoid salicin was higher in trees that had previously received the volatile bouquet of the herbivore-infested trees, and thus the volatiles of herbivore-damaged trees can be said to have a priming effect. Additionally, gypsy moth caterpillars avoided primed leaves and experienced reduced performance and increased mortality when forced to feed on them, possibly due to the priming of salicinoids. The results of a common garden experiment show that even slight changes in salicinoid concentration negatively affect the larval performance of the gypsy moth.

2.4 CHAPTER IV: INFLUENCE OF BLACK POPLAR DEFENSE RESPONSES ON A SPECIALIST HERBIVORE

Ontogenetic differences in black poplar (*Populus nigra*) leaf chemistry influence feeding and oviposition of the poplar leaf beetle *Chrysomela populi*

Thomas Fabisch, Jonathan Gershenzon & Sybille B. Unsicker

In preparation to be submitted to *Journal of Chemical Ecology*

This study investigated the influence of ontogenetic aspects of black poplar phytochemistry on feeding and oviposition of the specialist leaf beetle species *Chrysomela populi*. It also searched for consequences of the oviposition behavior of adult female beetles for their offspring. Additionally, feeding- as well as egg-induced changes in black poplars phytochemistry were tracked. Adult beetles preferred young leaves over slightly mature leaves for feeding, which was reflected in the higher concentrations of primary metabolites found in younger leaves. However, young leaves were avoided for oviposition, which mainly occurred on the slightly mature leaves. Although some ontogenetic differences in black poplar chemical composition might explain the observed oviposition behavior, the choice did not affect the larval development. Hence, the phytochemistry was not the only influential factor and other factors like shelter and competition are probably involved in the choice of feeding site as well. The application of egg material on young and slightly older black poplar leaves, respectively, induced the phytohormone salicylic acid and the specialized metabolite salicin. The induction of both compounds was weaker in the slightly mature leaves, which might influence aspects of egg or larval development.

3 MANUSCRIPTS

3.1 MANUSCRIPT I

SPECIFICITY OF HERBIVORE DEFENSE RESPONSES IN A WOODY PLANT,

BLACK POPLAR (*POPULUS NIGRA*)

Formular 1

Manuskript Nr. 1

Titel des Manuscriptes: Specificity of Herbivore defense responses in a Woody Plant, Black Poplar (*Populus nigra*)

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Der Kandidat / Die Kandidatin ist

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Status: publiziert

Anteile (in %) der Autoren / der Autorinnen an der Publikation

Autor/-in	Konzeptionell	Datenanalyse	Experimentell	Verfassen des Manuskriptes	Bereitstellung von Material
Fabisch T.	50	100	100	80	-
Unsicker S.B.	25			10	-
Gershenzon J.	25			10	100

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Specificity of Herbivore Defense Responses in a Woody Plant, Black Poplar (*Populus nigra*)

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Abstract
The specificity of woody plant defense responses to different attacking herbivores is poorly known. We investigated the responses of black poplar (*Populus nigra*) to leaf feeding by three lepidopteran species (*Lymantria dispar, Laothoe populi* and *Amata mogadorensis*) and two leaf beetle species (*Phratora vulgatissima* and *Chrysomela populi*). Of the direct defenses monitored, increases in trypsin protease inhibitor activity and the salicinoid salicin were triggered by herbivore damage, but this was not herbivore-specific. Moreover, the majority of leaf salicinoid content was present constitutively and not induced by herbivory. On the other hand, volatile emission profiles did vary among herbivore species, especially between coleopterans and lepidopterans. Monoterpenes and sesquiterpenes were induced in damaged and adjacent undamaged leaves, while the emission of green leaf volatiles, aromatic and nitrogen-containing compounds (known to attract herbivore enemies) was restricted to damaged leaves. In conclusion, indirect defenses appear to show more specific responses to attacking herbivores than direct defenses in this woody plant.

Keywords Specificity of plant defense · Salicinoids · Phytohormones · Herbivore-induced volatiles · Trypsin protease inhibitor

Introduction

Plant chemical defenses of many types are well known to be induced upon attack by insect herbivores. Such induction is sometimes thought to be specifically tailored to the attacking herbivore species giving rise to terms such as the *specificity of plant responses* (Karban and Baldwin 1997), the *specificity of elicitation* (Stout et al. 1998) and the *specificity of induced resistance* (Agrawal 2000). However, the causes and mechanisms of insect herbivore-specific responses in plants are not yet fully understood. Recent studies have investigated whether a plant responds in an herbivore-specific manner may dependent on the feeding guild of the insect, the level of feeding specialization (reviews by Ali and Agrawal 2012; Bonaventure 2014; Heidel-Fischer et al. 2014 and references therein) or salivary cues (Erb et al. 2012) and herbivore-associated microbe communities (Acevedo et al. 2015).

However, most investigations of the specificity of plant response to different insect attackers have focused on only a single defensive compound or compound group. For example, Van Zandt and Agrawal (2004) reported that the volume of pressurized latex, a putative anti-herbivore defense in milkweed, was differentially induced after herbivory when comparing monarchs (*Danaus plexippus)* to swamp milkweed beetles (*Labidomera clivicollis)*. Silva et al. (2017) observed differences in the profiles of tomato volatiles when comparing plants infested by the whitefly *Bemisia tabaci* to plants infested by the leaf miner *Tuta absoluta*. Studies of multiple classes of defenses and defense signals are uncommon.

Research on the specificity of plant defense induction has also concentrated on herbaceous rather than woody plant species, which are less studied due to the methodological problems accompanying their size and longevity (Lämke & Unsicker 2018). However, these characteristics of woody plants may lead to different responses to herbivores. First, throughout their longer lives woody plants may repeatedly encounter the same herbivore species. Second, they may be under constant attack during the growing season, from leaf flush (van Asch and Visser 2007) to senescence (White 2015). Third, among aboveground organs, the larger amount of biomass concentrated in stems may make losses of leaves to herbivores less critical. This might lead to a defense strategy where damaged tissue is sacrificed while defenses are

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concentrated in surrounding tissue. In light of these possibilities, both specific and non-specific defense responses might be viable strategies for woody plants under different conditions. While non-specific defenses are effective against more herbivores, a specific defense tailored to a single herbivore may be less costly (Onkokesung et al. 2016). However, there are comparatively few studies investigating the specificity of woody plant defense responses after herbivory, such as those conducted in willow (Fields and Orians 2006) and birch (Hartley and Lawton 1987). Yet these studies focus on a narrow set of defensive compounds, while investigations on a broad set of chemical compounds in combination with measurements of defense hormones are still missing.

The aim of this study was to investigate the defense responses of black poplar (*Populus nigra*) towards feeding by five different leaf-chewing insect herbivore species (two coleopterans and three lepidopterans) commonly occurring on poplar. We investigated herbivore-species-specific changes in the defense-related phytohormones salicylic acid (SA) and jasmonic acid (JA), salicinoids, trypsin protease inhibitor activity, and volatile organic compounds in black poplar to obtain a more complete picture about defense responses in woody plants. Salicinoids, a group of phenolic glycosides highly abundant in poplar trees (Boeckler et al. 2011) negatively affect generalist herbivore performance (Hemming and Lindroth 1995; Lindroth and Peterson 1988; Osier and Lindroth 2001). Protease inhibitors (Bradshaw et al. 1990; Haruta et al. 2001) and certain classes of volatiles (Clavijo McCormick et al. 2014b; Unsicker et al. 2015) are also typical poplar compounds reported to be active in defense against herbivores.

Among the herbivores, the two beetle species, *Chrysomela populi* (poplar leaf beetle) and *Phratora vulgatissima* (blue willow leaf beetle), and one of the lepidopteran caterpillar species *Laothoe populi* (poplar hawk moth) used in this study are specialist feeders, according to the classification by Ali and Agrawal (2012), because they feed on only a narrow range of tree species within the Salicaceae. In contrast the lepidopteran caterpillar species *Amata mogadorensis* and *Lymantria dispar* are true generalists, accepting host plant species from different plant families. Most of these herbivores may occur together on *P. nigra,* especially at the end of the season. In this study, we expected to find marked variations in the specificity of poplar defense responses both among various herbivore species and among different classes of defense metabolites. Since defoliation by chewing herbivores typically has only minor effects on the salicinoid concentrations of poplar (Boeckler et al. 2013, Osier and Lindroth 2001), we hypothesized that the induction of these phenolics would be weak and not herbivore species-specific. However, we expected protease inhibitor activity to be differentially induced, especially when comparing lepidopteran with coleopteran herbivores. Differential induction of protease inhibitors between these taxa has been described before (Chung and Felton 2011). It was also reported that the spectrum of volatile organic compounds induced by herbivores depends on feeding mode, the level of feeding specialization (Danner et al. 2018, Rowen and Kaplan 2016) and the composition of their oral secretions (Acevedo et al. 2015). We therefore hypothesized that volatile emission in black poplar would vary depending on the species identity of the attacker. In order to test these hypotheses, we investigated defense responses in both herbivore-damaged and nearby undamaged foliage.

Material and Methods

Plants and Insects

Populus nigra saplings were grown from cuttings of young trees made in the summer. All genotypes were originally taken from a natural black poplar population located in a floodplain forest on the Oder River of northeastern Germany (52°34′1" N, 14°38′3″ E). The trees were reared in the greenhouse under summer conditions (24 °C; 60% relative humidity; 16 hr/8 hr, light/dark) in 2-L pots filled with a 1:1 mixture of sand and soil. The experiments were carried out in a controlled environment chamber (20 °C/18 °C, day/night: 60% relative humidity; 16 hr/8 hr, light/dark) to which trees were transferred 24 hr before the start of the experiments. All trees were regularly fertilized and watered once per day.

Lymantria dispar caterpillars were hatched from eggs obtained from the US Department of Agriculture (Buzzards Bay, MA, USA), reared on artificial diet (MP Biomedicals LLC, Illkirch, France) in a climate chamber (23 °C, 60% relative humidity, 14 hr/10 hr, light/dark) and used in experiments as 3rd instar larvae. *Laothoe populi* caterpillars were obtained in 1st instar from a commercial provider (The World of Butterflies and Moths, UK, http://www.wobam.co.uk) and reared on black poplar foliage under laboratory conditions until they were used in experiments as 4th instar larvae. *Amata mogadorensis* caterpillars were hatched from eggs obtained from a private breeder (https://www.entomologenportal.de) and reared on black poplar foliage under laboratory conditions until they were used in experiments as 3rd instar larvae. The two beetle species *Chrysomela populi* and *Phratora vulgatissima* were reared from egg clutches collected in old-growth black poplar trees in the field.

Herbivore Treatments

To study the responses of black poplar to different herbivores, 40 young trees of a single tree genotype were selected. These trees, with a height of approximately 160 cm, were pruned to a

height of 80 cm four weeks before the actual experiment started to prevent them from growing too close to the light sources of the climate chamber in which the experiments were conducted. Starting from the pruned site, and counting in basal direction, 10 fully expanded leaves were then split in two sections of five leaves each that were separately enclosed with polyethylene terephthalate (PET) (Toppits Bratschlauch, Minden, Germany) bags fixed at both ends at the poplar stems with cable binders. Bags were left on throughout the duration of the experiment, and charcoal-purified air was pumped into and out of the bags through Teflon tubing at a flow rate of 0.5 L/min to prevent communication between the experimental plants via volatiles. The 40 young trees were split in groups of 10 plants each, resulting in four treatment groups. Three of these four groups received experimental leaf herbivory by one of three different herbivore species, *Lymantria dispar*, *A. mogadorensis* and *P. vulgatissima*. The insects were always released on the lower leaves (designated as "damaged leaves") with the upper uninfested leaves in the tree designated as "adjacent undamaged leaves" (Fig. 1). One group of 10 trees was not infested with any insects; the lower leaves of these trees served as controls for the damaged leaves, and the upper leaves as controls for the adjacent undamaged leaves. Ten third instar *Lymantria dispar* and *A. mogadorensis* caterpillars or 50 adult *P. vulgatissima* beetles were released on the lower leaves of the different herbivore treatment groups, and allowed to feed for 44 hr. After 24 hr, the number of caterpillars was reduced to six individuals per tree to avoid excessive leaf area loss. Due to space limitations in the controlled environment chamber, the experiment was split into three blocks, each representing an equal number of replicates from each treatment. Thus the first two blocks consisted of three representatives of each treatment and the third block of four replicates of each treatment, resulting in 12, 12 and 16 trees in each block respectively. The time lag between the subsequently processed experimental blocks was 2 days.

Herbivore Treatment for Volatile Collection

To study the emission of volatile organic compounds in response to herbivory, 50 trees of five different tree genotypes were selected. The trees had a height of approximately 80 cm and were younger than the trees in the experiment with a single tree genotype. Therefore, they were not pruned before the experiment but otherwise prepared as described in the previous section. Here, starting from the youngest fully developed leaf, 10 leaves in the basal direction along the stem, were selected to form two leaf pools as described in the previous section. The 50 young trees of five genotypes were evenly split in groups of 10 plants each, resulting in five groups containing 1–3 representatives of each genotype. Four of these groups received experimental leaf herbivory from one of four different herbivore species, *Lymantria dispar*, *Laothoe populi* (poplar hawkmoth), *P. vulgatissima* and *Chrysomela populi* (poplar leaf beetle). We used a different set of herbivores for this experiment because some of the species were collected in the field and are only available at certain times throughout the year. We have established laboratory cultures for *Lymantria dispar* and *P. vulgatissima* (see above), and thus these species were available for both experiments. The insects were released in the bag enclosing the lower leaves (Fig. 2). One group of trees did not receive any insects and thus functioned as the control group as described above. This experiment was conducted in two consecutive blocks, with 5 replicates of each of the five treatments (*Lymantria dispar*, *Laothoe populi*, *C. populi*, *P. vulgatissima*, non-damaged control) in a block. The time lag between blocks was 2 days. Five 3rd instar caterpillars of *Lymantria dispar* or *Laothoe populi*, 20 adults of *P. vulgatissima* or 5 adults of *C. populi* were released onto the lower leaves and allowed to feed for 44 hr. To avoid complete defoliation the number of individuals in the two caterpillar treatments was reduced to 3 one day after the experiment started.

Plant Harvest and Quantification of Experimental Leaf Damage

Right after the experiments, all damaged and adjacent undamaged leaves from all treated trees were harvested and photographed after being spread out on a white board with a reference area. After the midribs were removed (due to difficulties in consistently grinding them to a powder), leaves were flash-frozen in liquid nitrogen and then stored in 5 ml plastic vials at −80 °C until further processing. In addition, the equivalent leaves of non-damaged control trees were separately frozen. All leaf material was lyophilized (ALPHA 1–4 LDplus, Christ, Germany) and ground to a fine powder using a paint shaker (Scandex, Pforzheim, Germany) and five stainless steel balls (diameter 3 mm). Experimental leaf area loss in the different herbivore treatments was determined by analyzing the digital images of the leaves with Adobe Photoshop (Version 15.0.0, Adobe Systems Incorporated, San Francisco, USA) following the method described in Boeckler et al. (2013).

Defense Hormone Analysis

Defense hormones were extracted from an aliquot of 10 mg ground lyophilized leaf material. The aliquot was dissolved in 1 mL of pre-cooled methanol (MeOH) containing the following internal standards [D_6-abscisic acid (Santa Cruz Biotechnology, Dallas, TX, USA; 40 ng ml^{-1}), D_4-salicylic acid (Santa Cruz Biotechnology; 40 ng ml^{-1}), D_6-jasmonic acid (HPC Standards GmbH, Cunnersdorf, Germany; 40 ng ml^{-1}), ^{13}C-jasmonoyl-isoleucine (synthesis described

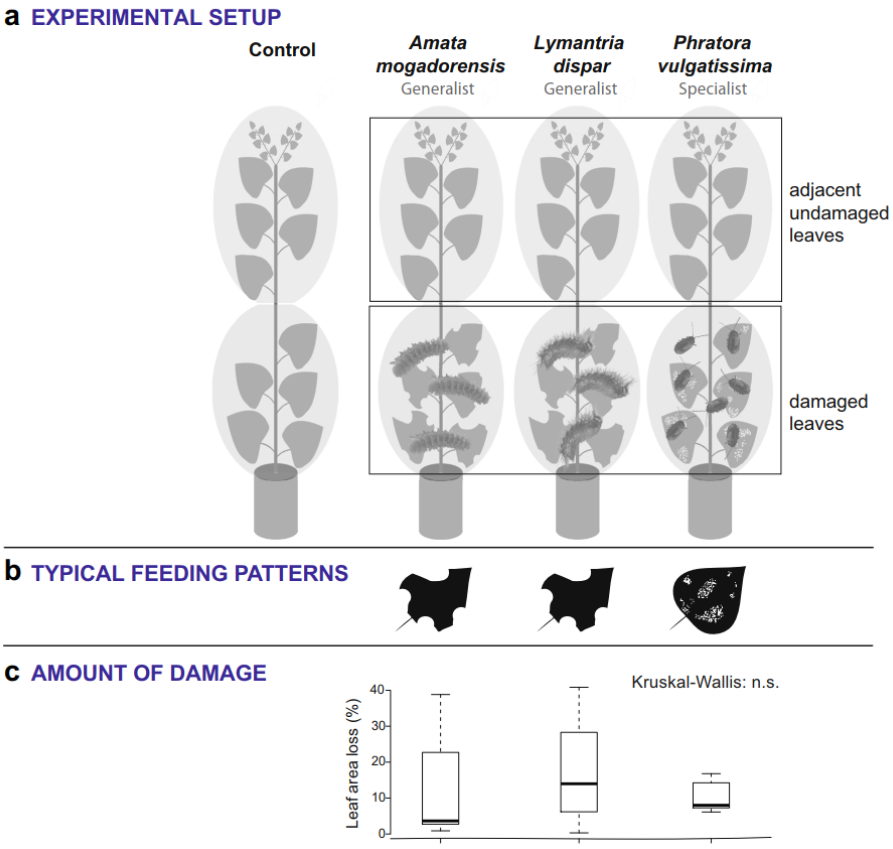

Fig. 1 Experimental design (**a**), typical herbivore feeding pattern (**b**) and amount of herbivore damage (**c**) for experiments in which each of three different herbivores was tested on leaves of single black poplar genotypes. The ten full-sized leaves of each sapling were divided into two groups of five leaves each. Each group was wrapped in a polyethylene terephthalate (PET) bag attached to the saplings with cable binders at both ends and supported with a constant flow of charcoal-purified air. The herbivores were caged on the lower leaves and allowed to feed for 44 hr. Lower leaves (from inside the cage) were harvested as "damaged" leaves. Upper leaves from the same sapling were sampled as "adjacent undamaged" leaves. Comparable leaves harvested in the lower and upper leaf pool of non-damaged trees (control) functioned as controls. Differences in the extent of herbivory were analyzed using the non-parametric Kruskal-Wallis test

in (Kramell et al. 1988), using ^{13}C-Ile, Sigma Aldrich; 8 ng ml^{-1})]. The samples were shaken for 30 sec with a paint shaker. Then they were centrifuged at 2000 g for 5 min, and 400 µL of the supernatant were transferred into a new tube. The rest of the supernatant was carefully removed from the solid phase using a pipette. Another 200 µL portion of the supernatant was used for salicinoid analysis. Subsequently, 1 mL of fresh MeOH (without labeled standards) was added to the solid phase before repeating the extraction procedure (shaker + centrifuge). Again, 400 µL (and 200 µL for salicinoids) of the supernatant was collected and combined with the supernatant of the first extraction. The extracts were stored at −20 °C until measurement.

Defense hormones were analyzed using high performance liquid chromatography (Agilent 1100 Varian ELSD, Varian, USA) coupled to a mass spectrometer (API 5000 LC/MS/MS System, AB Sciex, Framingham, MA, USA). The analytes were separated on a C18 column (XDB-C18, 50 × 4.6 mm × 1.8 µm, Agilent, Santa Clara, CA, USA) using a formic acid (0.05% in water) / acetonitrile gradient (flow: 1.1 ml min^{-1}) and detected via multiple reactions monitoring (MRM) in negative ionization mode (ion spray at −4500 eV at 700 °C) as described in Vadassery et al. (2012). Data were processed using Analyst 1.5.2 (Applied Biosystems, Foster City, CA, USA), and hormones were quantified relative to the peak area of their corresponding standard.

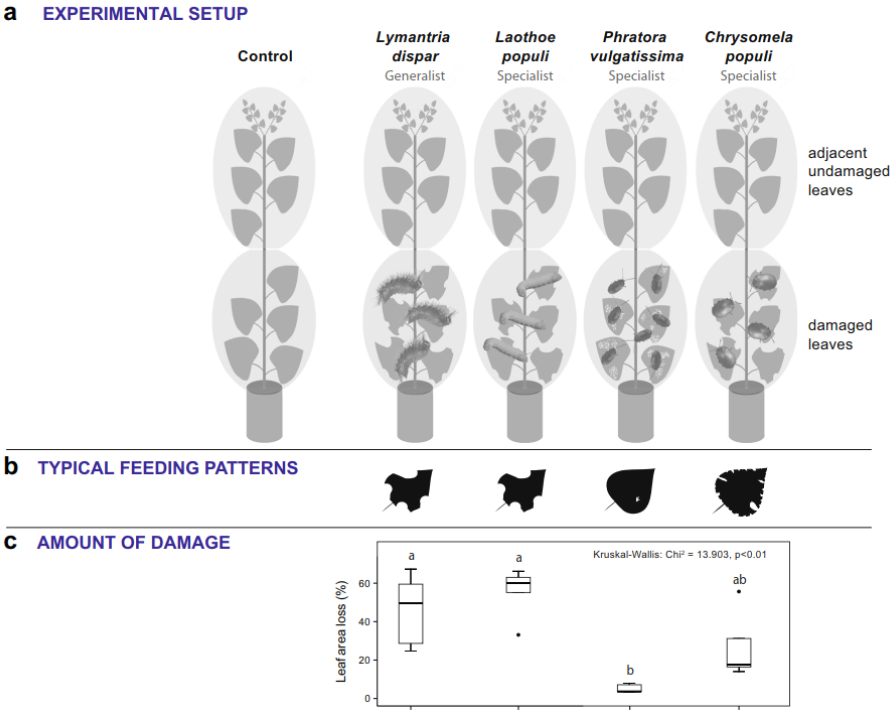

Fig. 2 Experimental design (**a**), typical herbivore feeding pattern (**b**) and amount of herbivore damage (**c**) for the experiment testing the effect of four different herbivore species on volatile emission of black poplar saplings of five different genotypes. The approximately ten full-sized leaves of each sapling were divided into two leaf pools containing five leaves each. Each leaf pool was surrounded by polyethylene terephthalate (PET) foil and supported with a constant flow of charcoal-purified air. The lower leaf pool was exposed to four different herbivore treatments, and leaves harvested as described in the Fig. 1 legend. Caterpillars of *Lymantria dispar, Laothoe populi*, and adults of *Phratora vulgatissima* and *Chrysomela populi* were used as herbivores. While *Lymantria dispar, Laothoe populi* and *C. populi* have a biting-chewing feeding mode, *P. vulgatissima* feeds in a piercing-chewing style and has therefore a different feeding pattern. All herbivores were allowed to feed for 44 hr. Differences in the extent of herbivory were analyzed using the non-parametric Kruskal-Wallis test. Pairwise comparisons were made using the *Dunn's* post hoc test. Black dots represent outliers

Trypsin Protease Inhibitor Activity

Protease inhibitor activity was analyzed via a radial diffusion assay (Jongsma et al. 1993). Samples of 10 mg of freeze-dried leaf material were dissolved in 400 µL of extraction buffer (25 mM Hepes, pH 7.2, adjusted with KOH, 3% PVPP, 2% PVP, 1 mM EDTA). After the addition of one steel ball (diameter 3 mm) and homogenization using a paint shaker (2 × 4 min), the samples were centrifuged at 4 °C and 2000 *g* for 10 min. A 200 µL portion of the supernatant was transferred into a 1 mL centrifuge tube and kept on ice until the analysis. An agar gel (1.8%) was prepared containing 2 µL/mL of fresh trypsin (Merck, Germany) dissolved in 25 mM Hepes-KOH buffer (pH 7.2). After pouring the gel solution onto a square petri dish, the gel was solidified for 3 hr at 4 °C. Subsequently, 5 mm-diameter wells were punched into the gel with a distance of 2 cm to each other using a hollow metal cork-borer. Along with the samples, a standard dilution series of bovine serum albumin (BSA) was added as reference. The gel was then incubated at 4 °C for 22 hr. After the gel was rinsed once with the extraction buffer (Hepes-KOH buffer) containing 10 mM $CaCl_2$ and stained with a solution of 72 mg Fast Blue B Salt in 90 mL Hepes buffer (25 mM, pH 7.2, pre-warmed to 37 °C), a 60 mg portion of *N*-acetyl-DL-phenylalanine beta-naphthyl ester (APNE) dissolved in 10 mL N, N-dimethylformamide was added before pouring the solution on the agar plate (pre-warmed to 37 °C as well). Incubation time was 90 min before the staining solution was decanted and the gel was rinsed with water, and a reference curve with BSA was created following the protocol of Bradford (1976) with assays run in triplicate. Before usage, the BSA was reconstituted by mixing with deionized water.

Salicinoid Analysis

Salicinoids were extracted during the procedure for the extraction of phytohormones (see above) with the addition of 0.8 mg/mL phenyl-β-glucopyranoside as an internal standard. The 2 × 200 μL extracts were combined and 400 μL of milli-Q-purified water was added before measuring the analytes via high performance liquid chromatography (HPLC). Analytes were injected onto a chromatographic column (EC 250 × 4.6 mm NUCLEODUR Sphinx RP, 5 μm, Macherey Nagel, Düren, Germany) connected to a precolumn (C18, 5 μm, 4 × 3 mm, Phenomenex). The temperature of the column oven was set to 25 °C. The mobile phase consisted of two solvents, solvent A (Milli-Q water) and solvent B (acetonitrile), from which solvent B was used in a gradient mode with time/concentration (min/%) of: 0:00/0; 19:00/52; 19:10/100; 21:00/100; 21:10/14; 26:00/14). The flow rate was set to 1 mL/min and injection volume to 20 μL. The signal was detected using photodiode array and evaporative light scattering detectors (Varian, Palo Alto, CA, USA). Using these settings and components, salicin eluted at a retention time of about 5.1 min, salicortin at about 10.2 min and homaloside D at about 15.2 min. The compounds were detected by absorption at 200 nm and identified by comparison of retention time in relation to those of standards isolated from previous work (Boeckler et al. 2013). Quantities were calculated on the basis of peak areas using standard curves prepared with pure standards corrected by the recovery of the internal standard.

VOC Collection and Analysis

VOCs released from various treatments were collected over a 4 hr period (9:00–13:00 hr) 40–44 hr after the insects were released on basal leaves of treated trees. VOCs in all treatments and leaf pools were trapped on five PDMS (polydimethylsiloxane) tubes (length: 5 mm) attached to 15 cm pieces of acetone cleaned aluminum wire hung inside each bag. PDMS tubes were prepared as described in Kallenbach et al. (2014). After the experiment tubes from each treatment and leaf pool were separately collected in glass vials (VWR International, Darmstadt, Germany) and frozen at −20 °C until further analysis.

Volatile analysis was performed with gas chromatography-mass spectrometry using the Ultra Thermo desorption unit TD20 connected to a quadrupole GC-MS-QP2010Ultra (Shimadzu, Kyoto, Japan). The PDMS tubes were placed in 89 mm glass TD tubes (Supelco, Sigma-Aldrich, Munich, Germany). After desorption in He with a flow rate of 60 mL/min at 200 °C for 8 min, the substances were cyro-focused onto a Tenax® adsorbent trap at −20 °C. The trap was then heated to 230 °C in 10 sec and the sample was injected into an Rtx-5MS column with a length/diameter of 30 m/0.25 mm and a film thickness of 0.25 μm (Restek, Bellefonte, PA, USA). Helium was used as carrier gas with a constant linear velocity of 44.3 cm/s. The TD-GC interface was held at 250 °C. The oven was set to 45 °C for 3 min, raised to 185 °C with an increase of 6 °C/min and subsequently to 320 °C at 100 °C/min with a 15 min hold. Electron impact (EI) mass spectra were recorded at 70 eV in scan mode from 33 to 350 *m/z* at a scan speed of 1666 Da/s. The ion source was held at 230 °C. Compounds were identified by comparison of mass spectra and retention times to those of authentic standards and spectra in Wiley and National Institute of Standards and Technology (NIST) libraries.

Statistical Analyses

Analyses were carried out using SPSS Statistics version 20.0 (IBM, New York, USA). For the volatile analysis using different poplar genotypes, genotypes with more than one replicate were analyzed as one genotypic replicate by taking the mean of the replicates. If necessary the dataset was log-transformed before statistical analysis. To analyze differences in the leaf chemical composition between all treatments, including the control plants, ANOVAs were used. To analyze differences in the leaf chemical composition only between the herbivore treatments ANCOVAs were used with the herbivore damage (leaf area loss) as a co-variable (compound ~ herbivore damage*treatment). Both the ANOVA and ANCOVA models were checked for homoscedasticity, outliers and normal distribution of residuals. For some compounds, the assumptions were violated and could not be rescued with data transformation. Here the treatment was analyzed using the non-parametric Kruskal-Wallis rank sum test. Posthoc comparisons were performed using the *Tukey-Kramer* post hoc test (for ANOVA) and *Dunn's* post hoc test (for non-parametric Kruskal-Wallis test). For the analysis, the experiment block was left out as well (even if its importance as a factor was significant) because the importance was based on the herbivore damage, which differed between the experiment blocks. Principal component analyses were performed using the online platform MetaboAnalyst (https://www.metaboanalyst.ca). Data were scaled, (mean-centered and divided by the standard deviation of each variable) and transformed using generalized logarithm transformation.

Results

Defense Hormones We assessed the levels of the defense-related phytohormones, salicylic acid (SA) and jasmonic acid (JA), in black poplar leaves from trees of a single genotype

after damage by three different herbivore species as compared to leaves from non-infested control trees. Protease inhibitor activity and salicinoids were all measured in samples collected in the same experiment, but volatiles were analyzed in a second, separate experiment. Concerning hormones, SA concentrations in damaged leaves did not differ among the various treatments, including those from non-damaged control trees (Fig. 3). However, JA levels in leaves damaged by *Lymantria dispar* and *P. vulgatissima* were significantly higher than the concentrations in the non-damaged control trees (*Dunn's* post hoc test: *Lymantria dispar* $P = 0.010$, *P. vulgatissima* $P < 0.001$). JA levels in *A. mogadorensis*-infested trees were not significantly different from the control trees, and there were no differences in JA levels among the three different herbivore species (Fig. 3).

In the adjacent undamaged leaves, SA concentrations did not differ between the four treatments (Fig. 3). However, there were significant differences between the four treatments in the JA concentrations of the adjacent undamaged leaves. Pairwise comparisons revealed that JA levels were significantly higher in the *P. vulgatissima*-infested trees (*Tukey-Kramer* post hoc test: $P = 0.015$) compared to the controls and also to the *Lymantria dispar*-infested trees (*Tukey-Kramer* post hoc test: $P = 0.012$). In contrast, the JA levels of *A. mogadorensis*-infested trees were not different from the controls and from the JA levels of the other herbivore-infested trees (Fig. 3). JA concentrations in the adjacent undamaged leaves were generally lower compared to those of damaged leaves.

Insect herbivory measured as % leaf area loss was integrated as a continuous variable in analyses of co-variance (ANCOVA – control trees excluded) to test the effect of leaf damage levels and insect herbivore species identity (main effect) on defense hormone concentrations in the damaged and adjacent undamaged leaves of *P. nigra*. In the damaged leaves SA concentrations were significantly affected by herbivore damage level but there was no significant effect of herbivore species identity (Table 1). JA concentrations in the damaged leaves were significantly affected by both herbivore damage level and herbivore species identity (Table 1).

In the adjacent undamaged leaves, JA concentrations were significantly affected by herbivore damage levels and herbivore species identity (Table 1). For SA, herbivore species identity had no effect on the concentration. The effect of herbivore damage levels on SA concentrations in the adjacent undamaged leaves could not be tested as the statistical assumptions for ANCOVA were not met.

Protease Inhibitor Activity We found significant differences in the activity of trypsin protease inhibitors (PI) in the damaged leaves of two of the three herbivore treatments as compared to

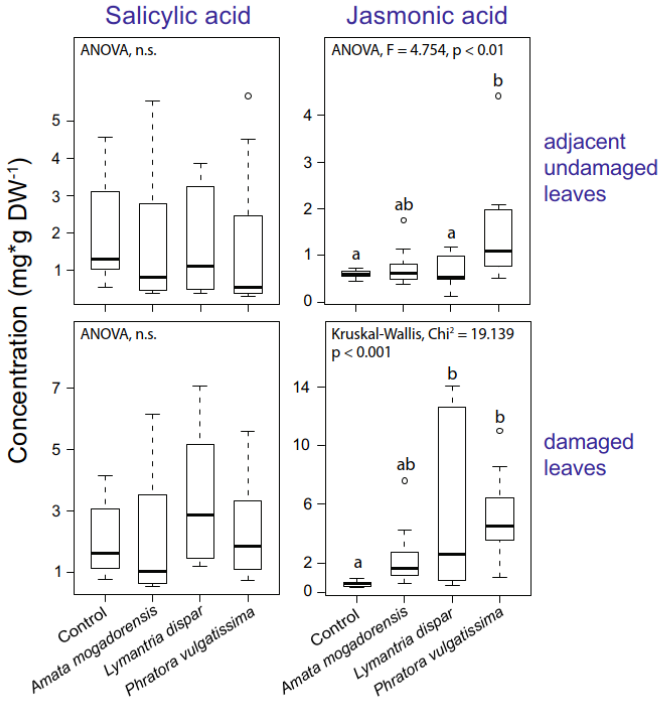

Fig. 3 Effect of damage by three herbivore species on the concentrations of two defense hormones, salicylic acid and jasmonic acid, in the damaged and adjacent undamaged leaves of young *Populus nigra* trees as compared to equivalent leaves from non-infested control trees. Samples were collected 44 hr after infestation with caterpillars of *Amata mogadorensis* and *Lymantria dispar*, and adults of the beetle *Phratora vulgatissima*, and from undamaged control plants. The boxplots depict medians ±1.5 x interquartile range of $n = 10$ tree replicates. Pairwise comparisons were conducted using *Tukey's* post hoc test (ANOVA) and *Dunn's* post hoc test (Kruskal-Wallis) and are indicated by small letters. Circles indicate outliers. Statistical results comparing only the herbivore treatments are given in Table 1

Table 1 Effect of herbivore damage level and herbivore identity on defense metabolites of young *Populus nigra* trees in damaged and adjacent undamaged leaves

	Herbivore damage level				Herbivore identity			
	df	df (error)	F-value	*p* value	df	df (error)	F / Chi$^{2\ (a)}$	p value
Damaged leaves								
Phytohormones								
Jasmonic acid	1	26	44.521	**<0.001**	2	26	7.106	**0.003**
Salicylic acid	1	26	13.792	**0.001**	2	26	0.451	0.642
Protease inhibitors								
Trypsin protease								
Inhibitor activity					2	26	4.901[a]	0.086[a]
Salicinoids								
Salicin					2	26	4.880[a]	0.087[a]
Salicortin	1	26	2.043	0.165	2	26	0.42	0.661
Homaloside D	1	26	1.855	0.185	2	26	1.002	0.381
Volatile organic compounds								
Monoterpenoids	1	14	0.157	0.698	3	14	1.883	0.179
Sesquiterpenoids					3	14	8.627[a]	**0.035**[a]
Aromatic volatiles	1	14	2.356	0.147	3	14	1.567	0.242
Nitrogenous volatiles	1	14	5.177	**0.039**	3	14	5.339	**0.012**
Green leaf volatiles					3	14	1.543[a]	0.672[a]
Adjacent undamaged leaves								
Phytohormones								
Jasmonic acid	1	26	8.816	**0.006**	2	26	8.126	**0.002**
salicylic acid					2	26	0.650[a]	0.722[a]
Protease inhibitors								
Trypsin protease								
Inhibitor activity					2	26	3.582[a]	0.167[a]
Salicinoids								
Salicin					2	26	0.003[a]	0.999[a]
Salicortin					2	26	0.235[a]	0.889[a]
Homaloside D					2	26	0.751a	0.687[a]
Volatile organic compounds								
Monoterpenoids	1	14	2.543	0.133	3	14	2.819	0.077
Sesquiterpenoids	1	14	0.242	0.630	3	14	1.157	0.361
Aromatic volatiles	1	14	2.709	0.122	3	14	2.537	0.099
Nitrogenous volatiles	1	14	0.008	0.929	3	14	0.342	0.796
Green leaf volatiles	1	14	2.195	0.161	3	14	2.173	0.137

Non-parametric Kruskal-Wallis and ANCOVA tests were employed to determine the significance of changes in the concentrations of the phytohormones salicylic acid and jasmonic acid, concentrations of salicinoids, levels of trypsin proteinase inhibitor activity, and emission of major groups of volatiles. The number of replicates was $n = 10$ trees for phytohormones, salicinoids and trypsin protease inhibitor activity and $n = 5$ trees for volatile organic compounds. The tests were performed on the same dataset shown in the graphs, but excluding the control treatment to check for differences only between the plants infested by the different herbivore species. Whenever the assumptions for ANCOVA were met, % leaf area loss (damage) was integrated as a covariate. When ANCOVA assumptions were not met, non-parametric Kruskal-Wallis tests were performed (marked by the letter "a"). Bold numbers indicate significant results

[a] Kruskal-Wallis H-Test

the controls, with the highest activity in the *P. vulgatissima* treatment (*Dunn's* post hoc test: *P. vulgatissima* $P = 0.004$, *Lymantria dispar* $P = 0.029$, Fig. 4). In the adjacent undamaged leaves, there were no significant differences in trypsin PI activity among treatments, but there was a trend for higher activity after *P. vulgatissima* herbivory (Fig. 4). Herbivore species identity did not significantly influence the PI activity in the damaged leaves although a trend was observed. In the adjacent undamaged

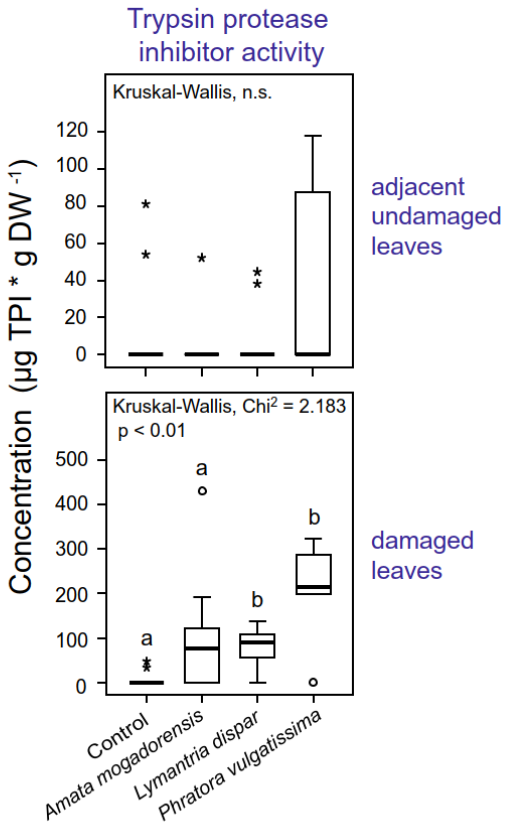

Fig. 4 Effect of damage by three herbivore species on the trypsin protease inhibitor activity in the damaged and adjacent undamaged leaves of young *Populus nigra* trees as compared to equivalent leaves from non-infested control trees. Samples were collected 44 hr after infestation with caterpillars of the two lepidopteran species *Amata mogadorensis* and *Lymantria dispar*, adults of the coleopteran species *Phratora vulgatissima*, and untreated control plants. The boxplots represent the median ± 1.5 x interquartile range of n = 10 tree replicates. Pairwise comparisons were conducted using *Dunn's* post hoc test (Kruskal-Wallis) and are indicated by small letters. Circles indicate outliers and asterisks indicate extreme outliers. The results of statistical analyses comparing only the herbivore treatments are given in Table 1

leaves, trypsin PI activity was not significantly influenced by herbivore species identity (Table 1). The influence of herbivore damage levels on trypsin PI activity could not be tested in either the damaged or adjacent undamaged leaves because assumptions for an ANCOVA were not met.

Salicinoid Concentrations Three different salicinoids, salicin, salicortin and homaloside D, were detected in black poplar leaves in this study. In the damaged leaves, we found significant differences in the salicin levels of the herbivore-infested trees as compared to the non-infested control trees (Fig. 5). Pairwise comparisons revealed significant inductions by all herbivore species versus the uninfested control trees (*Tukey-Kramer* post hoc test: *A. mogadorensis* $P = 0.010$, *Lymantria dispar* $P < 0.001$, *P. vulgatissima* $P < 0.001$), but no differences among the herbivore treatments were observed. Furthermore, we found no significant differences among treatments for salicortin and homaloside D (Fig. 5).

In the adjacent undamaged leaves, salicin levels were also significantly different in all herbivore-infested trees when compared to non-damaged control trees (*Dunn's* post hoc test: *A. mogadorensis* $P = 0.024$, *Lymantria dispar* $P = 0.008$, *P. vulgatissima* $P = 0.012$), and there were no significant differences among the herbivore treatments. The levels of salicortin and homaloside D were not significantly different when all treatments were compared (Fig. 5).

In the damaged leaves, herbivore identity did not significantly affect the concentration of salicin although a trend was observed. However, the influence of herbivore damage levels on salicin concentration could not be tested because ANCOVA assumptions were not met. Salicortin and homaloside D levels were not affected by herbivore damage levels or by herbivore identity (Table 1). Also, in the adjacent undamaged leaves the influence of herbivore damage level could not be tested as statistical assumptions were not met. However, herbivore species identity did not significantly affect the concentrations of the three salicinoids measured. (Table 1).

Volatile Organic Compounds To determine if different herbivore species cause different volatile responses in black poplar we set up a second experiment using multiple black poplar genotypes and a somewhat different set of herbivore species (Fig. 2). Phytohormone patterns in response to this set of insect herbivores were similar to the patterns observed in the first experiment (Fig. 3, Fig. S2). Altogether 86 volatile organic compounds were measured in this experiment, of which 69 could be (tentatively) identified (Table S1). A PCA performed with all identified volatiles measured in the headspace of the different treatments showed some separations between the herbivore treatments and the control treatment (Fig. S3). The volatile blends were further classified as monoterpenoids, sesquiterpenoids, green leaf volatiles (GLVs), aromatic compounds, nitrogenous compounds and "other volatiles" (compounds that did not fall into any of the chemical classes listed above), as we know from previous studies that certain volatile groups such as GLVs and nitrogenous compounds play essential roles in direct and indirect poplar defense.

Monoterpene emission from damaged leaves was significantly higher compared to emission from equivalent leaves on control trees (Fig. 6 lower row, *Tukey-Kramer* post hoc test: *Lymantria dispar* $P = 0.008$, *Laothoe populi* $P < 0.001$, *P. vulgatissima* $P < 0.001$, *C. populi* $P < 0.001$) but there were no significant differences found among the different herbivore treatments (Fig. 6 lower row). We also observed significant

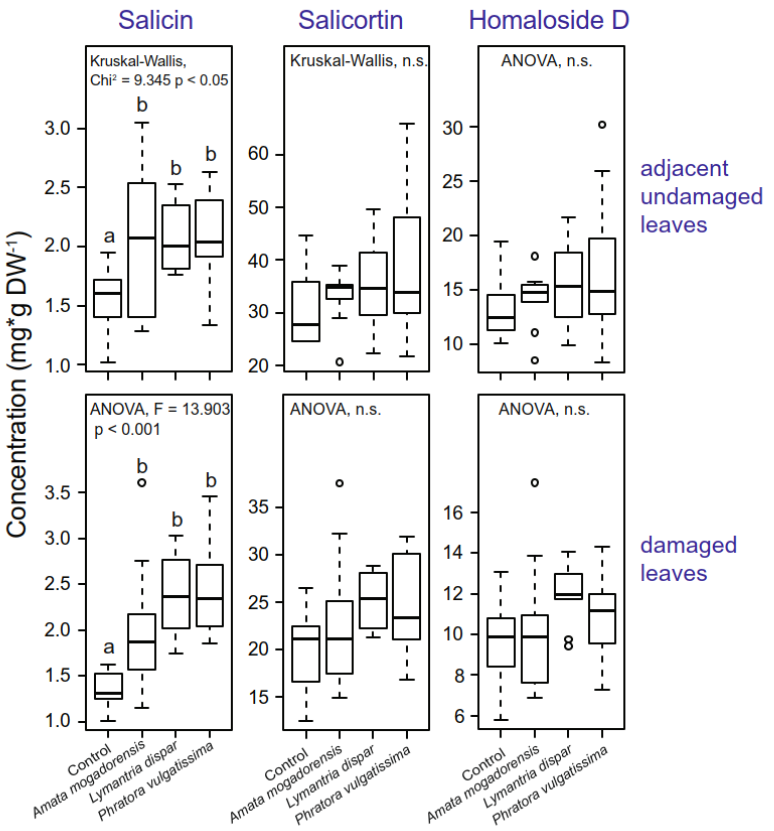

Fig. 5 Effect of damage by three herbivore species on the salicinoid concentrations in damaged and adjacent undamaged leaves of young *Populus nigra* trees infested by three different herbivore species as compared to equivalent leaves from non-infested control trees. Samples were collected 44 hr after infestation with caterpillars of two lepidopterans, *Amata mogadorensis* and *Lymantria dispar*, adults of one coleopteran, *Phratora vulgatissima*, and undamaged control plants. The box plots represent median ± 1.5 x interquartile range for n = 10 tree replicates. Pairwise comparisons were conducted using *Tukey's* post hoc test (ANOVA) and *Dunn's* post hoc test (Kruskal-Wallis) and are indicated by small letters. Circles indicate outliers. Statistical results comparing only the herbivore treatments are given in Table 1

increases in the emission of sesquiterpenes when comparing non-infested control trees with herbivore-infested trees, except for the *Lymantria dispar* infested trees (Fig. 6 lower row, *Dunn's* post hoc test: *Laothoe populi* $P = 0.044$, *P. vulgatissima* $P < 0.001$, *C. populi* $P = 0.001$). Additionally, significant differences in sesquiterpene emissions were found between *Lymantria dispar*- and *P. vulgatissima*-infested trees with the beetle *P. vulgatissima* inducing higher levels (*Dunn's* post hoc test: $P = 0.036$). A similar trend was observed between *Lymantria dispar*- and *C. populi*-infested trees (*Dunn's* post hoc test: $P = 0.086$). The emission of aromatic volatiles in the damaged leaves of all herbivore-infested trees was significantly increased compared to equivalent leaves on the control trees (Fig. 6 lower row, *Tukey-Kramer* post hoc test: *Lymantria dispar* $P = 0.026$, *Laothoe populi* $P = 0.049$, *P. vulgatissima* $P = 0.011$, *C. populi* $P = 0.001$), but no significant differences were observed among the different herbivore treatments. The emission of nitrogenous volatiles from damaged leaves was significantly increased in the beetle-infested trees (Fig. 6 lower row, *Dunn's* post hoc test: *P. vulgatissima* $P = 0.012$, *C. populi* $P = 0.001$). Their emission was not significantly different between the control trees and the trees infested by the two lepidopteran species *Lymantria dispar* and *Laothoe populi*. There were also significant differences in the emission of nitrogenous volatiles between trees infested by *Lymantria dispar* and trees infested by *C. populi* (Fig. 6 lower row, *Dunn's* post hoc test: $P = 0.028$). There were no significant differences in GLV emission from damaged leaves between the non-infested controls and any of the different herbivore treatments (Fig. 6 lower row). There were marginally significant differences in the damaged leaves with respect to the emission of "other volatiles", but posthoc comparisons did not show any significant differences among the treatments (Fig. S1).

From the adjacent undamaged leaves, monoterpene emission differed significantly among the treatments (Fig. 6, upper row). While the two caterpillar species (*Lymantria dispar* and *Laothoe populi*) did not significantly induce monoterpene emission as compared to the equivalent leaves on non-damaged control trees, the two beetle species did (*Dunn's* post hoc test: *P. vulgatissima* $P = 0.008$,

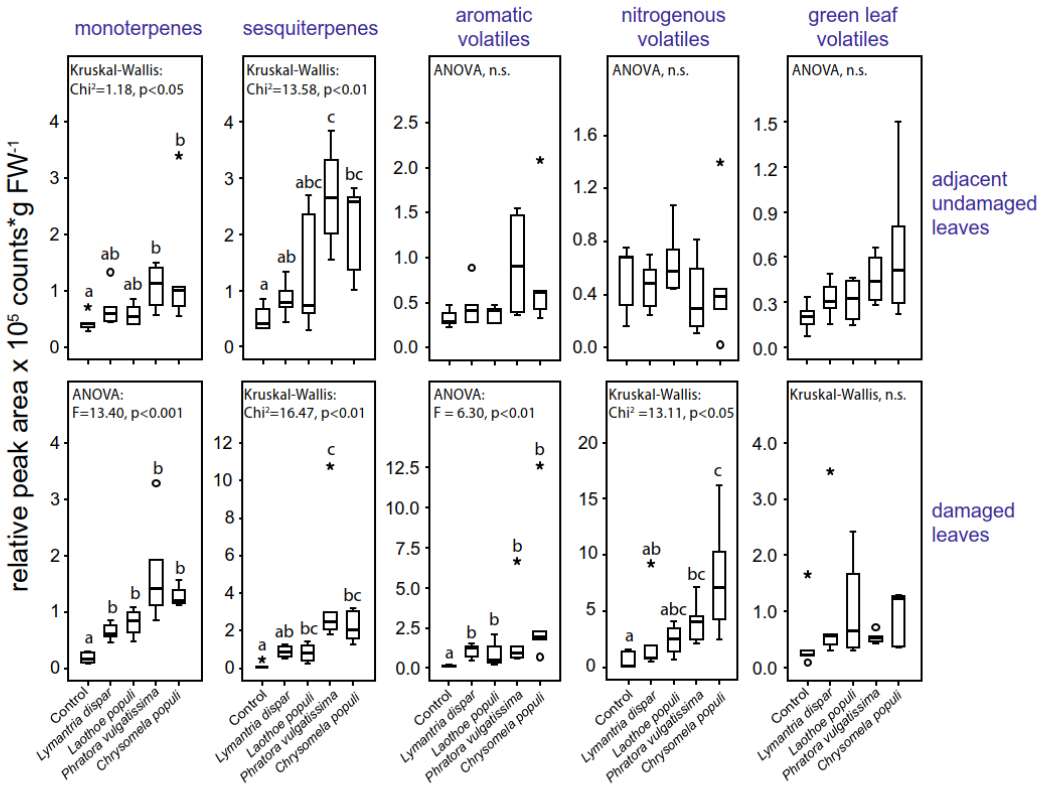

Fig. 6 Effect of damage by four herbivore species on the relative amounts of major groups of volatile organic compounds emitted from damaged (lower row) and adjacent undamaged leaves (upper row) of young *Populus nigra* trees as compared to equivalent leaves from non-infested control trees. Samples were collected 44 hr after infestation with caterpillars of two lepidopteran species, *Lymantria dispar* and *Laothoe populi*, adults of two coleopteran species, *Phratora vulgatissima* and *Chrysomela populi*, and untreated control plants. The box plots represent median ± 1.5 x interquartile range for $n = 5$ tree replicates. Pairwise comparisons were conducted using the *Tukey-Kramer* post hoc test (ANOVA) and *Dunn's* post hoc test (Kruskal-Wallis) and are indicated by small letters. Circles indicate outliers and asterisks indicate extreme outliers. Statistical results comparing only the herbivore treatments are given in Table 1

C. populi $P = 0.006$). Trends towards differences in monoterpene emission were also observed between *Laothoe populi*- and both beetle-infested trees (*Dunn's* post hoc test: *P. vulgatissima* $P = 0.062$, *C. populi* $P = 0.060$). Sesquiterpene emission in the adjacent undamaged leaves differed significantly between trees infested by beetles in the basal leaves and equivalent leaves on control trees (*Dunn's* post hoc test: *P. vulgatissima* $P = 0.002$, *C. populi* $P = 0.005$). In contrast, the two caterpillar species did not significantly induce sesquiterpene emission from undamaged leaves (Fig. 6, upper row). Differences in sesquiterpene emission were also observed between *Lymantria dispar*- and *P. vulgatissima*-infested trees (*Dunn's* post hoc test: $P = 0.032$). For aromatic and nitrogenous volatiles as well as for green leaf volatiles and other volatiles there were no significant differences among the treatments (Fig. 6 upper row, Fig. S1).

In the damaged leaves the emission of nitrogenous volatiles was significantly affected by the herbivore damage level and herbivore species identity, while sesquiterpene emission from damaged leaves was influenced by herbivore species identity (Table 1). In adjacent undamaged leaves none of the classified volatile groups was significantly affected by herbivore damage level and herbivore identity.

Discussion

In this study we found that young black poplar trees damaged by the three different leaf-chewing herbivores tested in the

single genotype experiment showed increases in the defense hormone jasmonic acid (JA), the salicinoid salicin and trypsin protease inhibitor activity. This was mainly observed in the damaged foliage, but in case of JA, also in the adjacent undamaged foliage. Additionally, all four herbivores tested in the second experiment induced different volatile organic compounds in the damaged as well as the adjacent undamaged foliage. While there was no herbivore-species-specificity for elicitation of the direct defenses surveyed, black poplar did display herbivore-specific emission of several classes of volatiles, in particular sesquiterpenes and nitrogenous compounds. In the case of sesquiterpenes the specificity of elicitation was also visible systemically in undamaged foliage adjacent to the attacked leaves.

When analyzing the two major defense-related phytohormones JA and SA, we found JA to be induced by the two leaf-chewing herbivores *Lymantria dispar* and *P. vulgatissima,* but not by *A. mogadorensis*. (Figure 3, Fig. S2). Local JA induction upon herbivore damage is a common phenomenon in herbaceous and woody plant species (Erb et al. 2012; Singh et al. 2016; Irmisch et al. 2014). The fact that SA was not induced by most of the herbivores investigated is in agreement with the literature. It is well documented that SA is mainly triggered by piercing-sucking insects like aphids (Li et al. 2016; Thaler et al. 2012) or infections by biotrophic pathogens (Kunkel and Brooks 2002). The general lack of SA induction by most of the herbivore species tested and induction of JA suggest a lack of specificity of defense signaling. The only exception was the specialist *Laothoe populi* that triggered the induction of SA in damaged leaves (Fig. S2). We also found that SA levels in damaged leaves were significantly affected by the amount of herbivore damage inflicted (ANCOVA, Table 1), even though there were no differences in SA concentrations between the different herbivore treatments (Fig. 3, Fig. S2). This result differs from other studies, where SA was not significantly influenced by chewing herbivores (Kawazu et al. 2012; Niveyro et al. 2013; Soler et al. 2012) although increasing and decreasing concentrations are also reported (Agrawal et al. 2014; Diezel et al. 2009). These observations demonstrate the complexity of the perception network involved in the recognition of herbivores by plants. This probably involves not only salivary cues, regurgitants and feces of herbivores, but also the associated herbivore microbiota. Investigations about the interaction of plants, herbivores and herbivore-associated microbes are just beginning and general models are hard to establish (Acevedo et al. 2015). The results obtained here and in other studies show that SA levels do respond to herbivory in a more subtle way than usually appreciated. The effects of the resulting signaling processes on the deployment of defenses are not known. Specificity might also be revealed by measurements of other hormones, such as ABA, ethylene and cytokinins (Erb et al. 2012), which were not quantified here.

Feeding by the generalist caterpillar species *Lymantria dispar* and one specialized leaf beetle, *P. vulgatissima*, increased the activity of trypsin protease inhibitors in damaged leaves (Table 1, Fig. 4). Also *A. mogadorensis* visibly increased the activity, although the differences were non-significant. The increased activity of protease inhibitors after wounding is a well-known inducible defense mechanism of plants (Jongsma and Bolter 1997). Since the production of protease inhibitors is associated with significant fitness costs (Zavala et al. 2004), their formation only in response to damage rather than being constitutively produced is understandable. Green and Ryan (1972) found the induction of protease inhibitors to be dependent on the number of wounding sites and the time after wounding. Although there were no significant differences in trypsin protease inhibitor activity in the leaves damaged by the different herbivore species, we observed a trend towards differential inductions (Table 1), which was probably caused by the higher numbers of wound sites from *P. vulgatissima* herbivory.

In contrast to most other black poplar metabolites measured, the major salicinoids, salicortin and homaloside D, were not induced by any of the herbivore species. A significant induction by leaf chewing caterpillars and beetles was only observed in the case of salicin (Fig. 5). Although there is little doubt about the role of salicinoids as defense compounds of Salicaceae plants (Boeckler et al. 2011), their induction patterns after herbivore attack are highly variable. While inductions of salicinoids are evident in some studies (Clausen et al. 1989; Fields and Orians 2006; Rubert-Nason et al. 2015; Stevens and Lindroth 2005) this is not always the case (Boeckler et al. 2013). The variability of herbivore-triggered salicinoid induction may arise because the levels of these phenolic compounds are influenced by many other factors. The most prominent factor is the genotype, which has been observed in many studies to cause much larger variation in salicinoid concentration than defoliation by herbivores (Osier and Lindroth 2001; Rubert-Nason et al. 2015). Other factors are the availability of nutrients and water (Hale et al. 2005) as well as organ, developmental and seasonal variation (Boeckler et al. 2011). Furthermore, individual salicinoids may be differentially induced after herbivory. The lower concentration of salicin compared to the other salicinoids measured does not necessarily mean that its defensive role is less important (Boeckler et al. 2016). In other species, inducible anti-herbivore metabolites with comparatively low concentrations but high impact on herbivores are known, such as indolic glucosinolates (Jeschke et al. 2016; Tian et al. 2005). Future studies should aim to investigate the toxicity and deterrency of herbivore-inducible salicin in comparison to the other less-inducible salicinoids.

When volatiles were measured, herbivory by two lepidopteran species and two leaf beetle species led to significant inductions of almost all major volatile groups (Fig. 6). The inducibility of plant volatiles after herbivory has been shown in both herbaceous (e.g. Fontana et al. 2009; Kigathi et al.

2013; Piesik et al. 2016; Skoczek et al. 2017) and woody plants (e.g. Courtois et al. 2016; Giacomuzzi et al. 2017; Maja et al. 2015) including poplar trees (Clavijo McCormick et al. 2014a; Philippe and Bohlmann 2007). In black poplar, nitrogenous volatiles released upon herbivory have been the focus of attention because they play a major role in attracting natural enemies of herbivores (Clavijo McCormick et al. 2014a). In other plant systems, terpenoids and GLVs are well-known to be involved in the attraction of natural enemies of herbivores (Turlings and Erb 2018). The induction of most of the groups of black poplar volatiles measured has been reported to be associated with JA signaling (Luck et al. 2016; Martin et al. 2003; Semiz et al. 2012). Herbivore-induced increases in protease inhibitor activity have also been connected with elevated jasmonate levels (Haruta et al. 2001; Lomate and Hivrale 2012). These reports are consistent with the JA induction measured in this study where we showed that an assortment of leaf-chewing herbivores all trigger increases in JA.

Elevated JA levels were found both in herbivore damaged leaves and in adjacent undamaged leaves (Fig. 3, Fig. S2) and the effect in adjacent undamaged leaves was dependent on the identity of the attacking herbivore species (Table 1). The systemic induction of JA in adjacent undamaged leaves after herbivory is a known phenomenon in herbs (Singh et al. 2016), but woody plants such as poplar have not always given consistent results. While herbivory by *Lymantria dispar* caused JA inductions exclusively in damaged poplar leaves (Clavijo McCormick et al. 2014b), other studies found JA also increased in the adjacent undamaged leaves (Babst et al. 2009; Boeckler et al. 2013). In the present study, *Lymantria dispar* feeding also led to significantly increased JA levels only in damaged leaves. A trend for higher JA levels in damaged leaves was also visible after feeding by the other generalist caterpillar species, *A. mogadorensis*. However, feeding by the beetle *P. vulgatissima* resulted in significantly higher amounts of JA in both the damaged and adjacent undamaged leaves (Fig. 3). We also observed significant systemic induction of salicin in the adjacent undamaged leaves of black poplar and of monoterpenes and sesquiterpenes as has been reported previously for this species (Clavijo McCormick et al. 2014b; Unsicker et al. 2015). In contrast the most prominent compounds induced only in herbivore-damaged leaves were the trypsin protease inhibitors and the nitrogen-containing volatiles (Fig. 4, Fig. 6). In herbaceous plants, herbivory commonly increases protease inhibitor activity significantly in both damaged and adjacent undamaged leaves (Arce et al. 2017; Bozorov et al. 2017; Lomate and Hivrale 2012). This is not true for poplar where induction in adjacent undamaged leaves (Bradshaw et al. 1990) has been reported to be much weaker and delayed compared to the induction in herbivore-damaged leaves (Haruta et al. 2001). Nitrogen-containing volatiles have previously been reported to be emitted only from herbivore-damaged foliage of black poplar and not systemically (Clavijo McCormick et al. 2014a; Unsicker et al. 2015). This may explain their use by herbivore predators and parasitoids as reliable cues to locate prey and hosts (Clavijo McCormick et al. 2014b).

The volatile bouquets released from black poplar upon herbivore damage differed between the lepidopteran and coleopteran species used in this experiment (Fig. 6, Fig. S3), especially for terpenoids, which were more abundant after coleopteran damage. Similar emission profiles of black poplar have been shown previously (Clavijo McCormick et al. 2014a, 2014b; Unsicker et al. 2015), even though a different volatile collection method was used here. In herbaceous plants, the emission of specific volatile patterns by different herbivore species is known (Cai et al. 2014; Danner et al. 2018; Hare and Sun 2011; Pinto-Zevallos et al. 2018; Turlings et al. 1998) and the pattern of stronger volatile induction after beetle herbivory was also observed (Hare and Sun 2011). At least one other woody plant also showed stronger induction of terpene emission after attack by coleopteran compared to lepidopteran herbivores (Moreira et al. 2013). Several possibilities might be responsible for herbivore species-specific defense responses in plants, including the type of damage and presence of specific elicitors (Ali and Agrawal 2012; Cai et al. 2014; Dicke et al. 2009; Rowen and Kaplan 2016). Specialist herbivores are thought to induce more total volatiles than generalists, although these patterns are not the same for each chemical class (Rowen and Kaplan 2016). One of the herbivore species employed in the present study can be classified as a generalist (*Lymantria dispar*) and the other three are specialists (*Laothoe populi, P. vulgatissima, C. populi*). However, the volatile pattern observed after herbivory differed more based on taxonomic grounds between lepidopterans and coleopterans than based on the degree of specialization.

In summary, our investigation demonstrated that both direct and indirect defenses are induced in black popular by a range of different herbivores. However, the induction of protease inhibitor activity (only in damaged leaves) and salicin (in both damaged and adjacent undamaged leaves) is not specific to the attacking herbivore species. Moreover, the bulk of salicinoids are constitutively present and do not change in concentration with attack. In contrast, the induced volatiles, of which some are known to play a role in indirect defense, do show specific responses to herbivores. The emission pattern from damaged and adjacent undamaged leaves differs between lepidopteran and coleopteran herbivores. Whether this pattern is characteristic of other woody plants requires further investigation.

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3.2 MANUSCRIPT II

POPLAR PROTEASE INHIBITOR EXPRESSION DIFFERS IN AN HERBIVORE SPECIES-SPECIFIC MANNER

Franziska Eberl[1], Thomas Fabisch, Katrin Luck, Tobias G. Köllner, Heiko Vogel, Jonathan Gershenzon & Sybille B. Unsicker

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Fabisch T.	40	40	50	10	-
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Eberl F.	40	40	50	80	-
Vogel H.		10			-
Luck K		10			-
Köllner T. G.	5				-

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Poplar protease inhibitor expression differs in an herbivore specific manner

Franziska Eberl[1*], Thomas Fabisch[1], Katrin Luck[1], Tobias G. Köllner[1], Heiko Vogel[2], Jonathan Gershenzon[1] and Sybille B. Unsicker[1]

Abstract

Background: Protease inhibitors are defense proteins widely distributed in the plant kingdom. By reducing the activity of digestive enzymes in insect guts, they reduce the availability of nutrients and thus impair the growth and development of the attacking herbivore. One well-characterized class of protease inhibitors are Kunitz-type trypsin inhibitors (KTIs), which have been described in various plant species, including *Populus spp*. Long-lived woody perennials like poplar trees encounter a huge diversity of herbivores, but the specificity of tree defenses towards different herbivore species is hardly studied. We therefore aimed to investigate the induction of KTIs in black poplar (*P. nigra*) leaves upon herbivory by three different chewing herbivores, *Lymantria dispar* and *Amata mogadorensis* caterpillars, and *Phratora vulgatissima* beetles.

Results: We identified and generated full-length cDNA sequences of 17 *KTIs* that are upregulated upon herbivory in black poplar leaves, and analyzed the expression patterns of the eight most up-regulated *KTIs* via qRT-PCR. We found that beetles elicited higher transcriptional induction of *KTIs* than caterpillars, and that both caterpillar species induced similar *KTI* expression levels. Furthermore, *KTI* expression strongly correlated with the trypsin-inhibiting activity in the herbivore-damaged leaves, but was not dependent on damage severity, i.e. leaf area loss, for most of the genes.

Conclusions: We conclude that the induction of KTIs in black poplar is controlled at the transcriptional level in a threshold-based manner and is strongly influenced by the species identity of the herbivore. However, the underlying molecular mechanisms and ecological consequences of these patterns remain to be investigated.

Keywords: Kunitz-type trypsin inhibitors; herbivore specificity; woody plants; tree defenses, Lepidoptera, Coleoptera, Salicaceae, Induced defenses, Proteinase inhibitors

Background

Over millions of years plants have developed numerous strategies to defend themselves against plant-feeding animals. Apart from indirect defenses, which involve the recruitment of an herbivore's natural enemies, plants can harm their attackers directly by producing mechanical barriers, chemical toxins and deterrents, or by using biochemical defenses that interfere with the herbivore's enzymatic machinery. Among chemical defenses, most emphasis has been placed on low molecular weight metabolites, but defensive proteins exist, such as protease inhibitors (PIs) that reduce the digestibility of plant tissue for the feeding herbivore. By inhibiting proteolytic enzymes in the midgut of the herbivore, PIs diminish protein digestion and hence lower the availability of free amino acids required for herbivore growth and development [15]. The PIs found in plants are numerous and diverse, with 99 different inhibitor families currently described [32]. Those PI families, as well as distinct members within a family, vary in their activity towards

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the four types of proteases found in herbivore guts, namely serine -, cysteine -, aspartic acid -, and metalloproteases. In herbivorous insects, the most abundant protein-degrading enzymes are the serine proteases [15]. It is therefore not surprising that serine PIs are widely distributed in the plant kingdom [20, 21]. One of the best characterized classes of serine PIs are the Kunitz-type trypsin inhibitors (KTIs; also Kunitz-type protease inhibitors, KPI), of which some are also able to inhibit cysteine proteases [2, 6]. KTIs are relatively small proteins with a mass of 20 to 25 kDa [39], with a β-trefoil structure, consisting of a β-barrel and several loops, of which one is binding to the active site of the target protease [42]. The biological activity of KTIs has been demonstrated by using gut extracts in in vitro assays [16, 29], as well as monitoring the fitness of herbivores feeding on KTI-enriched diets [2, 6, 22, 25, 26, 30]. Since the first description of a KTI in soybean [19, 24], most subsequent studies have also focused on KTIs from legume species [16, 17, 22, 30, 36, 43]. However, KTIs in trees have gained more attention in past years. In species of the genus *Populus*, several KTIs have been identified and characterized [7, 27, 29, 37, 39], and some shown to be inducible by mechanical wounding or insect herbivory [27–29, 39]. For example, feeding by the forest tent caterpillar, a generalist herbivore, increased *KTI* transcript abundance locally and systemically in hybrid poplar leaves [28]. In fact, genes encoding for KTIs belong to the most up-regulated ones in systemic poplar leaves upon mechanical wounding [9]. In a study by Philippe et al. [39] it was shown that the transcriptional induction triggered by wounding varies among the KTIs and in a time-dependent manner. So far, most studies used *Malacosoma disstria*, a generalist lepidopteran species, to investigate herbivore-triggered KTI responses in poplar [27, 28, 39]. To our knowledge, the specificity of poplar KTI induction towards other herbivore species has not yet been investigated.

Specificity of response to different herbivores may be especially important for large, long-lived woody perennials like trees, which encounter a vast diversity of herbivores in their lifetimes. For example, it is well known that plants react differently to leaf-chewing herbivores than herbivores feeding on phloem-sap [13, 23]. Specificity of anti-herbivore defenses can also be observed within the same feeding guild, and even within the same species depending on the insect's developmental stage. For example, early instar generalist caterpillars induced a stronger defense reaction in black poplar leaves than late instar caterpillars of the same species [31]. The underlying mechanism might be explained by HAMPs or DAMPS (herbivore- or damage-associated molecular patterns, respectively) that plants perceive when being attacked [13]. These are influenced by the physical attributes of herbivory, such as leaf area removal or the timing of tissue damage, but also by chemical cues such as salivary compounds of the herbivores [33]. All of these traits can be herbivore species-specific and may allow plants to distinguish among attackers and mount adequate and effective defenses against specific herbivores. In black poplar trees, such herbivore-specific reactions could be shown for signaling molecules [14], as well as chemical defense traits such as volatile emission [14, 31, 47]. In a recent study by Fabisch et al. [14], total PI activity against trypsin was more strongly induced by beetle feeding than by caterpillar feeding on black poplar leaves. However, to date, we do not know which specific genes are responsible for the observed differences in PI activity and whether or not transcription of PI-encoding genes differs between beetle- and caterpillar-fed leaves.

In this study, we therefore tested the hypothesis that different herbivore species induce *KTI* genes in a species-specific manner. We identified 17 *KTI* genes from a transcriptome of black poplar and generated full-length cDNA sequences of the most up-regulated ones. Gene expression patterns of these *KTI* genes as determined by qRT-PCR upon herbivory by three different insect species (Fig. 1) show striking differences among the species.

Methods

Plants and insects

Populus nigra L. (Salicaceae) trees were grown from cuttings obtained from trees in a common garden near Jena, Germany. These trees were originally derived from a single female genotype from a *P. nigra* population (species identified by Sybille Unsicker based on morphological features) located in Küstrin-Kietz, Germany (52°34′1" N, 14°38'3" E). Since cuttings for this study were taken from trees in a common garden, no permission was necessary for collecting plant material; a voucher specimen will be deposited in spring 2021 in the Herbarium Haussknecht (JE) in Jena, Germany. The cuttings were potted in 2 L pots, grown in the greenhouse (18/20 °C, night/day, relative humidity 60%, natural light with 9–14 h photoperiod, supplemented light for 12 h) and transferred to a climate chamber (18/20 °C, night/day; relative humidity 60%; photoperiod 16 h) 2 days before the onset of the experiment. Trees were either grown for 4 months to approximately 0.5 m (*Transcriptome samples*) or grown to a height of 1.6 m (approximately 6 months) and pruned back to 0.8 m 4 weeks before treatment (*Gene expression samples*).

Lymantria dispar L. (Erebidae, Lepidoptera) caterpillars are generalist feeders with a broad host range, preferably deciduous trees. *L. dispar* caterpillars were hatched from eggs kindly provided by the US Department of agriculture (USDA, Buzzards Bay, MA, USA)

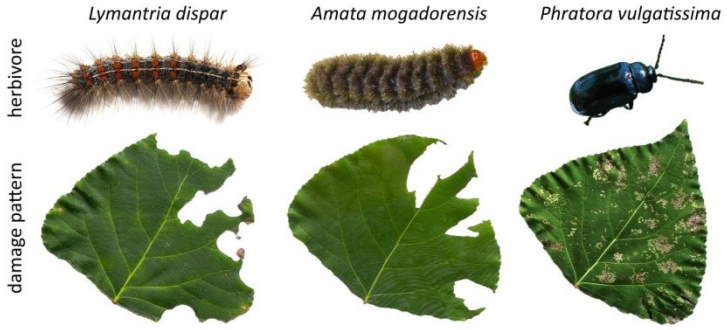

Fig. 1 Insects used in this study and their damage pattern after 2 d feeding on black poplar leaves. *Amata mogadorensis* and *Lymantria dispar* (gypsy moth) remove large areas from the leaves, whereas *Phratora vulgatissima* (blue willow beetle) causes small, but numerous lesions

and reared on artificial diet (MP Biomedicals LLC, Illkirch, France) in a climate chamber (14/10 h, light/dark, 20–23 °C, relative humidity 60%) until they reached the third instar, the stage used for the experiments. This species is reared continuously at the MPI-CE.

Amata mogadorensis Blachier (Erebidae, Lepidoptera) caterpillars are also generalists with a preference for woody plants and shrubs. *A. mogadorensis* caterpillars were hatched from eggs provided by a private breeder (www.entomologenportal.de) and reared on black poplar foliage until they reached the third instar, the stage used for the experiment. Individuals were reared until adult stage to confirm the species identity.

Phratora vulgatissima L. (Chrysomelidae, Coleoptera) beetles are specialists, feeding on a narrow range of hosts within the Salicaceae. Beetles (taxonomically determined by Lars Möckel; individuals in alcohol available at the MPI-CE) were reared in the laboratory on black poplar trees.

Experimental designs and sampling

Plant material from two different experiments was used to analyze the transcriptome (see *Transcriptome samples*) or the gene expression of Kunitz-type trypsin inhibitors (KTIs; see *Gene expression samples*).

Transcriptome samples

A leaf pool (8 leaves from the stem of a young black poplar tree ($n = 4$) was wrapped with gauze and then infested with *L. dispar* caterpillars (4 individuals per tree), adult *P. vulgatissima* beetles (6 individuals per tree), or left untreated (control). Due to time differences in the availability of the experimental insects, the beetle treatment was conducted two weeks earlier than the caterpillar treatment; both treatments had their own respective control group ($n = 4$), which was treated and sampled at the same time as the herbivore-treated plants, but was not exposed to herbivores. After 2 d, the treated leaves were flash-frozen in liquid nitrogen and stored at − 80 °C.

Gene expression samples

For gene expression analysis by qRT-PCR, leaf material from an experiment described in Fabisch et al. [14] was used, where further details on the methods are described. In short, a leaf pool (5 leaves) of black poplar trees ($n = 10$, but a random selection of 6 was used for gene expression analysis) was wrapped with PET bags (Bratschlauch, Toppits, Minden, Germany) and then infested with *L. dispar* caterpillars (10 per tree), *A. mogadorensis* (10 per tree), *P. vulgatissima* beetles (50 per tree), or left untreated (control). After 1 d, the number of caterpillars was reduced to prevent excessive leaf loss. After a total feeding period of 2 d, the leaves were photographed to assess the damage and subsequently flash-frozen in liquid nitrogen and stored at − 80 °C. The damage was quantified as leaf area loss from the photographs by reconstructing the original leaf area in the picture and counting the number of pixels representing the total and the removed leaf areas (Photoshop, Version 15.0.0, Adobe Systems Incorporated, San Francisco, USA). Pixels were converted to area (cm^2) using a reference field in the photograph.

RNA isolation and cDNA synthesis

Frozen leaves were ground in liquid nitrogen and RNA was isolated using the InviTrap Spin Plant Mini Kit (Stratec Biomedical AG, Birkenfeld, Germany), including DNase digestion. RNA concentration was measured with a NanoDrop 2000c spectrophotometer (Peqlab Biotechnologie GmbH, Erlangen, Germany). For transcriptome samples, an additional quality check was conducted with the RNA 6000 Nano Kit on a Bioanalyzer (Agilent, Santa Clara, CA, USA). cDNA was synthesized from RNA

using SuperScript-III reverse transcriptase and oligo-dT primers (Thermo Fisher Scientific, Waltham, MA, USA).

Transcriptome analysis

Sequencing was done at the Max Planck-Genome-Center (Köln, Germany) on a HiSeq 2500 (Illumina, San Diego, CA, USA) with 9 Mio reads per sample. Detailed information on quality control measures, the assembly of the de novo transcriptome and the annotation can be found in Eberl et al. [12], but the most relevant information will be summarized here. The annotation was done using, among others, BLAST, Gene Ontology (GO) and InterPro terms (InterProScan, EBI). Contigs encoding for potential KTI proteins were identified based on a positive BLAST hit against a known KTI in the NCBI nr database, GO terms associated with serine proteinase inhibitors and/or a hit against the Pfam domain PF00197 (Kunitz STI protease inhibitor), or InterPro domains IPR011065 (Kunitz inhibitor STI-like superfamily) and IPR002160 (Proteinase inhibitor I3, Kunitz legume). In order to identify further KTI candidates, the *P. nigra* transcriptome was uploaded in an internal database and used for BLAST analysis of poplar KTI sequences from NCBI (www.ncbi.nlm.nih.gov/) and Phytozome (https://phytozome.jgi.doe.gov/pz/portal.html). Digital gene expression analysis was carried out using CLC Genomics Workbench v9.1 to generate BAM (mapping) files, and expression levels were then estimated using QSeq Software (DNAStar Inc., Madison, WI, United States). The log2 (RPKM) values (normalized mapped read values; geometric means of the biological replicate samples) were used to calculate fold-change values. Differentially expressed genes were identified using the Student's t-test (as implemented in Qseq) corrected for multiple testing using the Benjamini–Hochberg procedure to check the false discovery rate (FDR). With an FDR-corrected *p*-value less than 0.05 a gene was considered significantly differentially expressed.

In addition to the *KTI* gene sequences in the transcriptome of the herein described experiment, another *KTI* gene (*PnKTI B1*) was identified from an additional leaf transcriptome from the same *P. nigra* genotype and comparable *L. dispar* herbivory treatment (unpublished). Furthermore, another sequence encoding a KTI (*PnKTI A4*, or SQ33325–2), which was not present in the transcriptome, was identified during amplification from cDNA (see below) with primers originally designed for *PnKTI A13* (SQ33325).

Cloning and sequencing of PI genes

Full-length open reading frames (ORF) were amplified from a mix of cDNA originating from herbivore-induced samples in a PCR using Phusion High Fidelity polymerase in HF-buffer according to the manufacturer's manual (New England Biolabs GmbH, Frankfurt/Main, Germany). Primers were designed based on the putative ORF from the transcriptome whenever available, or with the ORF of the homologous genes retrieved from the NCBI data base (https://www.ncbi.nlm.nih.gov/). PCR products were cloned into a PCR4-blunt TOPO vector (Thermo Fisher) and fully sequenced using the Sanger protocol and capillary sequencing with an ABI Prism-Gene- Analyser 3130xl (Applied Biosystems).

Sequence alignments and phylogenetic analysis

Homologs of *P. nigra KTI* sequences were identified using the BLAST-search of the NCBI data base (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and the *P. trichocarpa* genome v3.0 (https://phytozome.jgi.doe.gov/). Alignments and similarity calculations were done with Geneious software (Biomatters, Auckland, New Zealand).

An amino acid alignment of poplar KTI proteins was constructed using the MUSCLE algorithm implemented in MEGA6 [46]. Tree reconstruction was done with MEGA6 using the Neighbor-Joining method and the JTT matrix-based method. All positions with less than 80% site coverage were eliminated.

Gene expression analysis by qRT-PCR

cDNA (diluted 1:3 with water) from the *Gene expression samples* was used for quantitative real-time PCR (qRT-PCR), which was performed in a Brilliant III Ultra-Fast SYBR reaction mixture (Agilent) on a CFX Connect Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) with 40 2-step cycles (95 °C, 30s + 60 °C, 30s) and a melting curve from 53 to 95 °C. Primer sequences can be found in Table S2. The PCR products were verified by cloning and sequencing as described above. Gene expression was calculated using CFX Manager 3.1 (Bio-Rad) using the $\Delta\Delta c_q$ method and taking primer efficiencies into account. Values were normalized to *Actin* as a reference gene [41] and expressed relative to a control sample.

Trypsin-inhibiting activity assay

In order to correlate gene expression with protease inhibitor activity, the trypsin-inhibiting activity assay was performed as described in Fabisch et al. [14]. In short, 10 mg freeze-dried leaf material was extracted with 400 µL buffer (25 mM Hepes-KOH, pH 7.2, 3% PVPP, 2% PVP, 1 mM EDTA) and the extract tested for trypsin-inhibiting activity in a colorimetric (cleavage of N-acetyl-DL-phenylalanine beta-naphthyl ester) in-gel diffusion assay.

Statistical analysis

All data were checked for statistical assumptions, i.e. homogeneity of variances and normal distribution. Gene

expression data for all *KTI* genes had to be $\log_{10}$-transformed to meet the statistical assumptions for parametric testing. For gene expression data, a one-way MANOVA (multivariate analysis of variance) coupled to a Tukey's post-hoc test was applied. All statistical analyzes were conducted using SPSS 17.0 (SPSS, Chicago, IL, USA).

Results

Identification of herbivore-induced Kunitz-type trypsin inhibitors

The transcriptome of black poplar leaves with and without herbivory by two different insect species, *Lymantria dispar* (Lepidoptera) and *Phratora vulgatissima* (Coleoptera), was used to identify genes encoding herbivore-induced Kunitz-type trypsin inhibitors (KTIs). Among all sequences in the transcriptome, 45 were identified as protease inhibitor genes (PIs), of which 30 were up-regulated upon both caterpillar and beetle herbivory, seven showed different regulation patterns depending on herbivore identity, and eight were down-regulated upon herbivory by either of the herbivores (Table S3). Among the 45 PI genes, 15 belong to the *KTI*s, and were all up-regulated upon herbivory (Fig. 2). These 15 *KTI* sequences, plus two additionally identified *KTI* genes, were compared to previously described poplar *KTI*s (Table S4) and named according to the nomenclature of Ma et al. [27].

A phylogenetic analysis based on the amino acid alignment revealed that the KTIs cluster into 4 subfamilies (Fig. 3). Most of the 17 KTIs belong to the subfamilies A and C, whereas only one protein belongs to subfamily D. Interestingly, all members of the C-subfamily showed a low expression and were only marginally up-regulated upon herbivory in comparison to members of the other three subfamilies (Fig. 2). Therefore, KTIs from the subfamily C were not considered in further analysis. Out of the remaining *KTI* genes, those with the highest expression levels in herbivore-induced samples were chosen for cDNA sequencing, yielding the full-length open reading frames of ten *PnKTI* genes (Fig. 3).

Herbivore-specific induction of KTI gene expression

To study the specificity of *KTI* gene expression, we used three different herbivore species that exhibit either similar (*L. dispar, Amata mogadorensis*) or different (*P. vulgatissima*) damage patterns on black poplar leaves (Fig. 1), but all cause similar leaf area loss (Table S1). In a previous study, we showed that total trypsin inhibitor activity in black poplar leaves is induced upon herbivory by three different herbivores, especially by *P. vulgatissima* [14]. To study this phenomenon at the transcriptional level, the relative gene expression of nine candidate *PnKTI*s was analyzed by qRT-PCR, using randomly selected samples from this previous study.

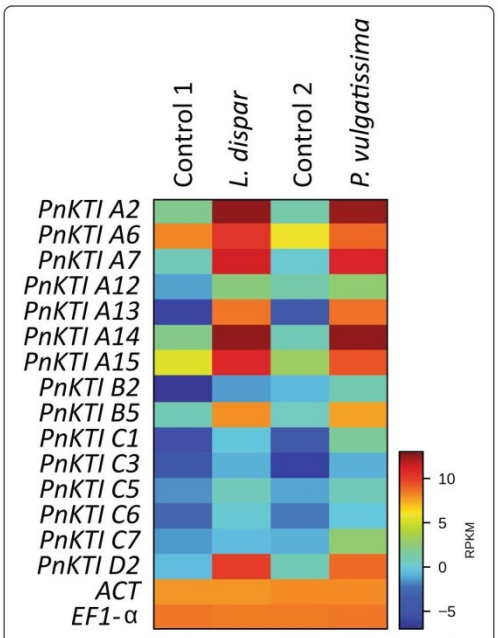

Fig. 2 Expression of Kunitz-type trypsin inhibitor (*KTI*) genes in black poplar leaves after feeding by gypsy moth caterpillars (*L. dispar*) or blue willow beetles (*P. vulgatissima*) compared to their respective controls (Control 1 and 2), and compared to *actin* (*ACT*) and *elongation factor 1-α* (*EF1-α*) as constitutively expressed 'housekeeping genes'. Shown are the mean RPKM (reads per kilobase of transcript per million mapped reads; $n = 4$) as result of the transcriptome analysis

While *PnKTI A2* could not be amplified in the qPCR reaction and was therefore excluded from further analysis, all of the remaining eight *PnKTI* genes showed significant up-regulation upon herbivory by all of the tested insects (Fig. 4). A multivariate analysis including damage severity as covariate revealed that the herbivory treatment had the strongest effect on *PnKTI* gene expression ($F_{(24)} = 7.230$; $P < 0.001$).

Constitutive expression levels in undamaged leaves differed among the *PnKTI* genes, with members of the A subfamily generally displaying higher expression levels than those of the B and D subfamilies (Table S5). Upon herbivory, however, the genes showed even more apparent differences in their inducibility (Fig. 4). Caterpillar herbivory by *L. dispar* and *A. mogadorensis* resulted in an up-regulation of all *KTI* genes by approximately 10 (*PnKTI A6*) to 2000-fold (*PnKTI D2*) in comparison to the constitutive levels. All *KTI* genes were induced to

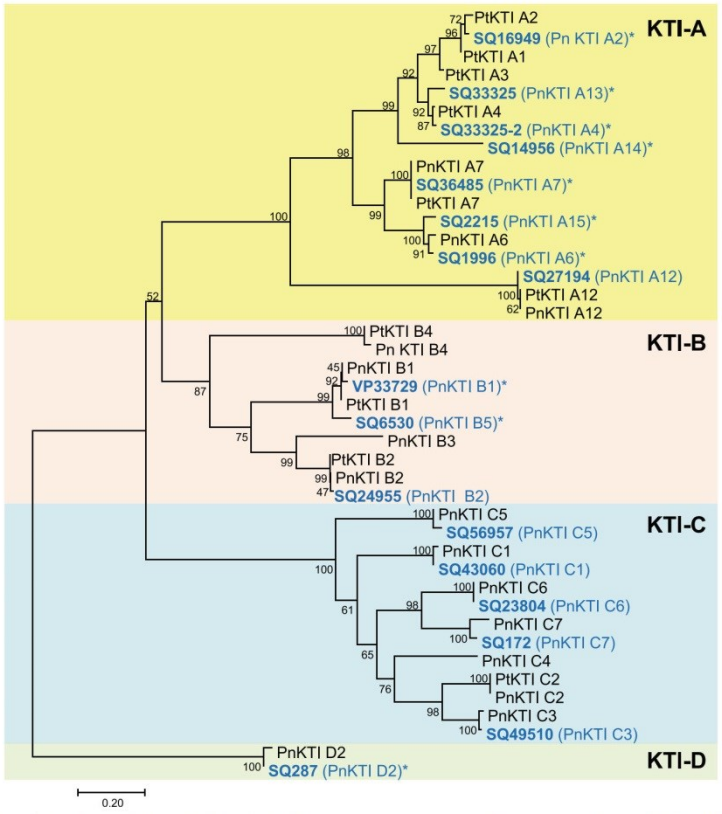

Fig. 3 Phylogenetic tree of poplar KTI proteins. PnKTIs identified in this study are shown in blue and asterisks mark full-length cDNA sequences. The tree was inferred using the Neighbor-Joining method and the JTT matrix-based method. Bootstrap values ($n = 500$) are shown next to each node. The tree is drawn to scale, with branch lengths in the units of the number of amino acid substitutions per site

similar levels by these two lepidopteran herbivores. Beetle herbivory by *P. vulgatissima*, however, caused a much stronger induction of *KTI* gene expression than caterpillar herbivory. Expression levels in beetle-damaged leaves increased up to 40,000-fold (*PnKTI D2*) compared to undamaged controls. Nevertheless, the induction levels differed substantially among the individual genes, ranging from approximately 40 (*PnKTI A6*) and several hundred (*PnKTI A15*, *B1*, *B5*) up to several thousand-fold (*PnKTI A13*, *A14*, *D2*). Interestingly, *PnKTI D2*, a gene with one of the lowest constitutive expression levels, showed by far the strongest relative induction upon both caterpillar and beetle herbivory (Fig. 4h).

When considering herbivore treatments only (excluding the undamaged control group), we found that the damage severity (% leaf area loss) did not have a substantial effect on expression levels of most of the *PnKTI*s. Only two genes, *PnKTI A7* and *PnKTI B1*, were significantly influenced by this factor in their expression (ANCOVA; *PnKTI A7*: $F_{(1)} = 9.348$, $P = 0.009$; *PnKTI B1*: $F_{(1)} = 5.012$; $P = 0.042$). Accordingly, the expression of the *PnKTI*s also did not correlate with the damage severity, except for *PnKTI A7*, which showed a positive relationship with leaf area loss (Spearman's rank correlation: $\rho = 0.556$, $P = 0.017$). The total trypsin-inhibiting activity (Table S1 [14];), on the other hand, strongly correlated with the expression of all *PnKTI*s with a positive relationship (Table 1).

Discussion

Here we describe sequence analyses and expression patterns of Kunitz-type trypsin inhibitors (KTIs) in black poplar (*Populus nigra*), including ten full-length cDNA sequences, of which six had not been described before in

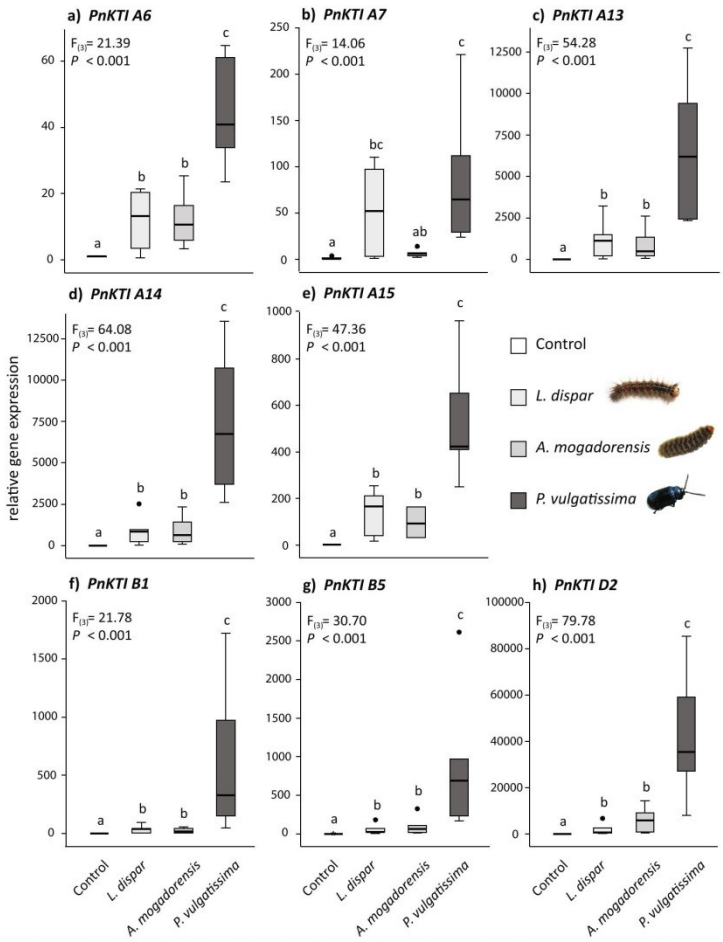

Fig. 4 Transcript accumulation of Kunitz-type trypsin inhibitor genes (*KTIs*) of the **a**, **b**, and **d** subfamily in black poplar leaves after herbivory by two caterpillar species (*L. dispar, A. mogadorensis*) and one beetle species (*P. vulgatissima*). Shown are the gene expression normalized to *Actin* and relative to a control sample as boxplots (median with upper and lower quartile as bars; $n = 6$); results of the ANOVA are given in each graph. Different letters indicate significant differences among groups ($P < 0.05$; Tukey's post-hoc test)

P. nigra. Eight of these PnKTIs were studied in the context of herbivore species-specific induction patterns in leaves and we could show that beetle herbivory elicits a much stronger transcriptional response than caterpillar herbivory of the same magnitude.

Expression levels and inducibility of individual black poplar KTI genes

The up-regulation of protease inhibitor (PI) transcription and activity has been described previously, also in black poplar [27, 38]. However, both constitutive expression levels and amplitude of induction vary between studies. In our study, members of the C-subfamily generally showed low expression levels and little or no up-regulation upon herbivory (Fig. 2). In contrast, Ma et al. [27] observed stronger herbivore induction for most of the genes in this subfamily. This suggests that the regulation of *KTI* transcription depends on more factors than herbivore feeding or wounding alone. Certain traits of the plants, such as age, genotype [44] or previously experienced damage may play a role, but also the experimental conditions such as abiotic conditions,

Table 1 Correlations of individual *PnKTI* gene expression versus total foliar trypsin-inhibiting activity (μg g^{-1} DW; data from [14]) in all herbivore-treated (*L. dispar*, *A. mogadorensis*, and *P. vulgatissima* feeding) samples of black poplar leaves. Spearman rank-correlation, significant values are highlighted in bold font

PnKTI	Spearman's ρ	*P*
PnKTIA6	0.707	**0.001**
PnKTI A7	0.648	**0.004**
PnKTI A13	0.646	**0.004**
PnKTI A14	0.730	**0.001**
PnKTI A15	0.700	**0.001**
PnKTI B1	0.710	**0.001**
PnKTI B5	0.597	**0.009**
PnKTI D2	0.582	**0.011**

timing [27, 39] or damage severity could potentially influence expression levels. However, there are also consistent patterns among the different studies. In our study, *PnKTI D2*, the only member of the D subfamily, showed the highest inducibility, i.e. relative change upon herbivory (Fig. 4). The same gene was amongst the most up-regulated *KTI*s upon herbivory and mechanical wounding in another black poplar study [27]. Similarly, the high herbivore-induced expression levels of *PnKTI A14* in our experiments (Fig. 2; Table S5) match well with the results obtained for the corresponding ortholog in a hybrid poplar species (*P. trichocarpa* x *deltoides*) after herbivory and mechanical wounding [39]. However, this gene also showed relatively high transcript abundance in undamaged controls, assuming also a role in constitutive defense or primary metabolism. On the contrary, *PnKTI D2*, which displays minimal expression levels in undamaged tissue in our and a similar study [27], seems to act exclusively in induced anti-herbivore defense.

There was no correlation between gene expression for most of the *PnKTI* genes and damage severity, which suggests a threshold-based activation of *PnKTI* transcription rather than continuous control, in which more damage would lead to higher *KTI* transcript levels. Furthermore, we found a strong positive relationship between the trypsin-inhibiting activity in poplar leaves and the transcription levels for all *PnKTI* genes. This indicates that *P. nigra* KTI activity is predominantly controlled at the transcriptional level and hence by de novo biosynthesis. The importance of de novo biosynthesis of stress-induced PIs has already been demonstrated in rice [40].

Herbivore specificity in PnKTI induction

When we analyzed the transcription of *KTI*s in leaves damaged by different insect herbivores, it became evident that beetles elicited a much stronger induction of all tested *KTI*s than caterpillars (Fig. 4). Similar observations come from pine trees [35] and milkweed [1, 48], where beetle herbivory induced stronger defense responses (resins and terpenes, or latex, respectively) compared to caterpillar herbivory. Species-specificity has been reported for the induction of PIs in other systems, though not in poplar trees. In soybean, damage by fall armyworm caterpillars increased the activity of PIs, whereas thrips damage did not [43]. De Oliveira et al. [11] even observed varying response of tomato PIs to damage by herbivores of the same genus. They showed that PI activity was induced by the spider mite *Tetranychus urticae*, but was suppressed by *T. evansi* [11]. Interestingly, feeding damage by lepidopteran and coleopteran herbivores in tomato yielded opposite results to our study in black poplar. Here, gene expression and trypsin inhibiting activity was more strongly induced by the tobacco hornworm than by the Colorado potato beetle [10].

The difference in *PnKTI* expression between beetle (*Phratora vulgatissima*) and caterpillar (*Lymantria dispar* and *Amata mogadorensis*) herbivory might be based on the different damage pattern these insects cause, even though all three of them are leaf chewers and removed the same total leaf area. While caterpillars removed large chunks of the leaves, the beetles caused small but numerous lesions in the leaves (Fig. 1). The number of lesions was found to be a key factor determining the emission of volatiles, another important anti-herbivore defense trait in black poplar [31]. Other factors, such as the duration of damage or the chemical compounds deposited on the plant may also be important. When artificial damage was administered to lima bean with a mechanical caterpillar, changes in the amount of time that damage lasted as well as the area damaged affected the emission of volatiles [34]. Furthermore, species-specific compounds in the saliva could trigger distinct defense responses or the magnitude of response as reported here. The importance of insect-derived elicitors for PI induction has been demonstrated in another poplar species, where mechanical wounding and simultaneous application of oral secretions from forest tent caterpillars suppressed the induction of PIs [39]. It is likely that oral secretions of the insects used in this study also exhibit a suppressive effect, maybe with varying efficacy on PI induction. Whether herbivore host range, comparing generalists such as *L. dispar* and *A. mogadorensis* versus specialists such as *P. vulgatissima*, plays a role in the induction of PIs, is not clear. Specialists usually possess a higher tolerance towards specific chemical defenses of their hosts, such as salicinoids in black poplar trees [3]. An increased induction of a defense, such as the PIs, to specialist herbivores could therefore be a more effective way to defend against these

insects. Future studies using more herbivore species, or generalists and specialists that are more closely related to each other and cause similar feeding patterns, are necessary to determine if herbivore host range influences PI induction.

Whether the herbivore specific induction patterns of *PnKTI*s have ecological relevance is another open question. One factor that plays an important role in this context is 'effect specificity' [20]. PIs possess varying effectiveness in defense against different herbivores, as could be observed in the performance of five different herbivores that had been reared on PI-supplemented diets [8]. Similarly, the cotton bollworm exhibited distinct preference and performance towards different classes of protease inhibitors [25]. This can be explained by the fact that PIs, on the one hand, vary in their ability to inhibit different proteases, i.e. trypsin, chymotrypsin and elastase [29], and that insects, on the other hand, vary in their gut protease activities [8, 20]. Additionally, the gut pH, which differs substantially between Lepidoptera and Coleoptera [20], also influences the inhibitory activity of PIs [49]. It would therefore be interesting to dissect the role of individual KTIs in black poplar towards different insect herbivores, for example by using transgenic trees or diet supplementation of recombinant KTIs. 'Response specificity' towards herbivore species is believed to be more cost-effective for a plant than a similar response to all herbivores [20]. Keeping in mind the fitness costs that are linked to the biosynthesis of PIs [18], a plant might aim to induce a subset of PIs to which a herbivore is most sensitive. In this context, PI activity should not be evaluated independently of other plant defense compounds. In tobacco, PIs function synergistically with the chemical defense compound nicotine, which becomes more toxic when herbivores have to compensate for nutritional deficits by increased feeding activity [45]. Black poplar contains toxic defense compounds called salicinoids, which have been shown to negatively influence herbivore performance and survival [4, 5]. Therefore, possible synergistic effects between salicinoids and PIs, the two main components of direct defense in this tree should be investigated in future studies.

Conclusion

Our major conclusion is that PI induction in black poplar leaves depends on the identity of the feeding herbivore, with beetles inducing a stronger response than caterpillars. Furthermore, PI activity is regulated at the level of transcription and most likely in a threshold-based fashion. However, most of the molecular mechanisms underlying the patterns observed and their ecological consequences remain to be elucidated.

Abbreviations

KTI: Kunitz-type trypsin inhibitors; qRT-PCR: Quantitative real-time PCR; cDNA: Complementary DNA; PI: Protease inhibitor; HAMPs: Herbivore-associated molecular patterns; DAMPs: Damage-associated molecular patterns; ORF: Open reading frame; PCR: Polymerase chain reaction; MANOVA: Multivariate analysis of variance

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12870-021-02936-4.

Additional file 1: Table S1. Feeding damage by the three herbivores and trypsin-inhibiting activity in poplar. **Table S2**. Primer sequences used for cloning and qRT-PCR. **Table S3**. Differential expression of contigs annotated as protease inhibitors in the transcriptome of black poplar leaves. **Table S4**. Nomenclature of KTI homologs in this and other studies. **Table S5**. Quantification cycles of the qRT-PCR analysis for individual *KTI* genes.

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Authors' contributions

FE, TF, JG, TGK, SBU conceived the project; TF conducted the biological experiments and leaf sample collection; FE analyzed the gene expression; KL and FE cloned and sequenced all candidate genes; TGK created the phylogenetic tree; HV assembled and analyzed the transcriptome; FE wrote the manuscript; all authors reviewed and commented on the manuscript. The authors read and approved the final manuscript.

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Availability of data and materials

The short-read data have been deposited in the EBI short read archive (SRA) with the following sample accession numbers: ERS5844847- ERS5844862. The complete study can also be accessed directly using the following URL: http://www.ebi.ac.uk/ena/data/view/PRJEB43369.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Publisher's Note

3.3 MANUSCRIPT III

VOLATILE MEDIATED DEFENSE PRIMING IN BLACK POPLAR.

MINOR CHANGES CAN CAUSE MAJOR DIFFERENCES

Sandra Lackner, Thomas Fabisch, Heiko Vogel, Beathe Rothe, Jonathan Gershenzon & Sybille B. Unsicker

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Autoren: Sandra Lackner, Thomas Fabisch, Heiko Vogel, Beate Rothe, Jonathan Gershenzon, Sybille B. Unsicker

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Anteile (in %) der Autoren / der Autorinnen an der Publikation

Autor/-in	Konzeptionell	Datenanalyse	Experimentell	Verfassen des Manuskriptes	Bereitstellung von Material
Fabisch T.	25	20	20	10	-
Unsicker S.B.	10			2,5	-
Gershenzon J.	5			2,5	100
Lackner S.	60	70	80	85	-
Vogel H.		5			
Rothe B.		5			

_____ _____

Unterschrift Kandidat/-in Unterschrift Betreuer/-in (Mitglied der Fakultät)

Title: Volatile mediated defense priming in black poplar. Minor changes can cause major differences

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Abstract

In recent years evidence arose that herbivore-induced plant volatiles (HIPVs) can prime neighboring plants for increased defense upon possible future attack. However, the actual ecological consequences are not well studied for woody plants. The goal of this study was to elucidate whether volatile-mediated defense priming in black poplar would increase plant fitness. We analyzed phytohormones, transcriptional changes and multiple defense compounds in HIPV exposed poplar leaves. Additionally we conducted a food choice and a performance assay with gypsy moth caterpillars. HIPV exposure had no effect on phytohormones, transcriptional changes or protease inhibitors. Caterpillars avoided HIPV exposed leaves and showed an increased mortality and decreased performance when feeding on exposed leaves. We show that salicinoids are primed upon HIPV exposure and argue that the increase in salicinoids is responsible for reduced larval performance. Our results suggest that volatile mediated defense priming leads to increased fitness in black poplar.

Introduction

Upon herbivore attack plants release herbivory-induced plant volatiles (HIPV) for direct and indirect defense (reviewed in Unsicker *et al*., 2009). Additionally those volatiles can function as signals between undamaged parts of the same plant or neighboring plants (Heil and Silva Bueno, 2007). The volatile receiving tissue or plant, which is now aware of a possible danger close-by, can prime their defense for a potential attack (Li *et al*., 2012). Volatile-mediated defense priming does not lead to phenotypical changes. It will only become aware after a subsequent herbivore attack through a more rapid and/or intense reaction compared to a normal non-primed plant. Therefore priming is believed to increase plant fitness since it would decrease the level of damage and additionally it is argued that the costs of priming are lower compared to induced defenses (van Hulten *et al*., 2006; Kost, 2006; Douma, 2017).

Volatile-mediated priming can lead to an increase in terpenes (Engelberth *et al*., 2004), an increase in non-volatile plant defenses like protease inhibitors (PI) (Tscharntke *et al*., 2001) and/ or an upregulation of defense related genes, shown in most of the studies mentionend below. This phenomenon has been described mainly for herbaceous plant like lima bean (Heil and Silva Bueno, 2007; Kost and Heil, 2006; Yi *et al*., 2009), Arabidopsis (Godard *et al*., 2008), maize (Engelberth *et al*., 2004; Ton *et al*., 2007; Erb *et al*., 2015), wheat (Ameye *et al*., 2015) and tobacco (Paschold *et al*., 2006). The literature for woody plants is sparser in general but there are some reports of priming in trees like alder (Tscharntke *et al*., 2001) and aspen (Li *et al*., 2012).

It has been shown for birch that inter- and intra-plant signaling is possible even at the same time (Girón-Calva *et al*., 2014). For perennial plants intra-plant priming might be of a greater ecological importance due to the short-lived nature of most volatile organic compounds under natural conditions and the fact that volatiles can move freely were there a restrictions in the vascular system (Karban *et al*., 2006; Frost *et al*., 2007 and 2008; Li and Blande, 2017).

However many open questions remain regarding the priming phenomenon like which are the priming volatile compounds? What is the mechanism of the signal transduction and does priming have an actual consequence on feeding herbivores? Here we show that in black poplar (*Populus nigra*) the exposure to HIPVs does not influence phytohormones, transcriptional changes, PIs, free sugars nor total protein content but primes salicinoids only. We demonstrate that gypsy moth (*Lymantria dispar*) caterpillars actively avoid primed leaves and show higher mortality and reduced performance when forced to feed on primed leaves. Furthermore we argue that the increase in salicinoids causes the reduction in larval

performance.

Materials and Methods

Tree and insect rearing

Black poplar (*Populus nigra*) trees were grown monoclonal from stem cuttings of two genotypes growing in a common garden near Jena. All greenhouse experiments were carried out with the genotype "f65", only the performance assay was carried out with a different genotype (f169). Stem cuttings were grown in 2 l pots filled with substrate mixture (55 % Klasmann-Tonsubstrat, 25 % Klasmann S1 [Klasmann-Deilmann GmbH, Geeste, Germany], 15 % sand and 5 % expanded shale) were grown and maintained in a greenhouse under summer conditions (24 °C, 60 % relative humidity, 16 h / 8 h light cycle) up to 1 – 1.20 m height until the start of the experiments.

Gypsy moth (*Lymantria dispar*) caterpillars were hatched from egg clutches and reared on artificial wheat germ diet (MP Biomedical, Eschwege, Germany) in a climate chamber (25 °C, 60 % humidity, 14:10 L:D period) until the start of the experiments.

Volatile exposure of receiver trees

The trees, both emitters and receivers, were cloaked in a PET bag (perimeter 62 cm, height according to tree height; Bratschlauch, Toppits, Minden, Germany). At 1.8 l min^{-1} air was pushed over an air purifying charcoal filter into the emitter bag (poplar infested with 10 4^{th} instar *L.dispar* caterpillars). Via a diaphragm pump (Laboport, KNF Neuberger, Freiburg, Germany) the headspace was then transported from the bottom of one emitter to the top of three receivers using a three-way split at 0.5 l min^{-1} each. In total 1.5 l min^{-1} was pulled from the emitter resulting in a slight overpressure in the bags to avoid contamination from the outside. The PET bags and all hoses (Teflon tubing, 4mm in diameter) required for the air transport were fixed with cable ties. Receiving trees were exposed to emitter volatiles for 48 h. Control treatments were treated accordingly.

Volatile collection of the emitters and analysis

After the first 24 h of the 48 h of volatile transmission from emitting to receiving trees, volatile emissions from the emitter trees were collected for 2 h with Poropak traps (Alltech, Florida, USA). Following the collection the traps were eluted twice with 100 µl dichlormethane containing an internal standard (nonyl acetate, concentration = 10 ng μl^{-1}, Sigma Aldrich, Seelze, Germany). For identification and quantification of compounds we used GC-MS and GC-FID, for more detailed information see

supplementary material.

Subsequent herbivore treatment of HIPV exposed receiver trees

Directly after the 48 h of HIPV exposure the air connection was removed and 10 4th instar *L. dispar* caterpillars were allowed to feed on the caterpillar and the HIPV exposed + caterpillar treatment (Fig. 1) for an additional 24h. Control and HIPV exposed treatments were resting for 24 h, resulting in total 72 h of experiment After 72 h the caterpillar food choice assay was carried out and 5 middle-aged leaves were harvested and stored for analyses.

Salicinoid and Catechin analyses

Phenolic compounds were extracted from 10 mg of freeze-dried *P. nigra* leaf material with 1 ml methanol containing an internal standard (0.8 mg ml^{-1} phenyl-β-glucopyranoside, Sigma Aldrich, Seelze, Germany). Extracts were diluted 1:2 with Milli-Q water before separation by an HPLC (1100 Series, Agilent Technologies, Santa Clara, CA, USA) equipped with a reversed phase column (EC 250/4.6 Nucleodur Sphinx, RP 5µm, Macherey-Nagel, Düren, GER). The mobile phases consisting of two solvents, solvent A (Milli-Q water) and solvent B (acetonitrile), were run with solvent B in gradient mode. The time/concentration (min/%) of the gradient was set to 0/14; 22.00/58; 22.10/100; 25.00/100; 25.10/14; 30.00/14 with a constant flow rate of 1 ml min^{-1}. The column oven temperature was set to 25 °C. The signal was detected with Photo Diode Array (PDA) and Evaporative Light Scatter (ELSD) detectors (Varian, USA). Concentrations were calculated on the basis of the peak areas as described in (Boeckler *et al*., 2013).

Other analyses

Phytohormones, phenylacetaldoxime and free sugars were extracted in parallel with the phenolic compounds and analyzed on HPLC-MS (for more information see Vadassery *et al*., 2012; Irmisch *et al*., 2013 and Eberl *et al*., 2020; respectively). For more details on RNA extraction and transcriptome analysis see supplemental. Protease inhibitor concentration was determined *via* a radial diffusion assay as described in (Lackner *et al*. 2019).

Caterpillar food choice assay

In a choice assay 19 2nd instar *L. dispar* caterpillars were offered leaf discs from either control or HIPV exposed plants to see whether they can distinguish between the treatments. Four leaf discs (16 mm Ø)

of each treatment were stuck alternatingly on pins glued to a 90 mm petri dish equidistantly. A moist filter paper was placed at the bottom of each arena to avoid a drying of the leaf discs. One caterpillar per arena was then allowed to feed on the leaf discs for 24 h. Afterwards, the discs were photographed and herbivore damage was determined using Adobe Photoshop CS5 (Adobe, San Jose, CA, USA) as described in Boeckler et al. (2013).

Caterpillar performance assay

To investigate whether HIPV exposure affects caterpillar development, a performance assay was conducted. Therefore, groups of 7 2nd instar *L. dispar* larvae were forced to feed on either HIPV exposed or control leaves, 12 groups each. Caterpillar groups were monitored and weighed over ten days. Dead larvae, if traceable, were removed before weighing. Each group was held in a 135 mm petri dish. Offered leaves were cut from pretreated trees and supplied with water. Leaves were removed from experiment, harvested for analysis and exchanged with fresh leaves simultaneously according to larval feeding behavior, namely after 4, 6 and 9 days. Prior to the assay trees treatment were exposed to HIPVs for 48 h, as described above. The performance assay was then started directly after the 48 h of exposure. So at the time of leaf harvest, the exposure was respectively 4, 6 or 9 days ago.

Common garden caterpillar performance experiment

The effect of salicinoid concentration on the performance of young *L. dispar* larvae was studied in a common garden experiment under natural conditions in June 2016. The trees originally derived from monoclonal stem cuttings of a natural black poplar population located in a floodplain forest along the Oder River of northeastern Germany (52°34’1” N, 14°38’3” E). Nine trees of different genotypes, which vary naturally in salicinoid concentration, were selected for the experiment. On each tree one branch was selected. Starting from the youngest fully developed leaf and counting in basal direction 8 young leaves were enclosed with a net bag, fixed on both ends with cable binders. Subsequently, twenty 2nd instar *L. dispar* larvae were released into the leaf pool. The larvae were allowed to feed for 14 days. After 14 days caterpillars were weighed. Dead larvae were not considered in the analysis. Afterwards all leaves were harvested and shock-frozen in liquid nitrogen. In the lab all leaf material was lyophilized (ALPHA 1-4 LDplus, Christ, Germany) and stored at -20 °C until further analysis.

Statistical analysis

Random forest analysis was carried out using the metaboanalyst webservice (Chong, J. and Xia, J. 2018)

with following parameters: number of trees = 5000, number of predictors = 5. The OOB error was 0.125. All other statistical analyses were done with IBM SPSS Statistics version 25 (SPSS, Chicago, IL, USA). All data were checked for statistical assumptions such as normal distribution, heterogeneity of variances and sphericity. In case of two group comparisons t-tests or related samples Wilcoxon rank tests were performed. ANOVA followed by Tuckey post hoc comparison was performed in case of normally distributed data with homogeneous variances. In case of non-parametric data, Kruskal-Wallis tests followed by Dunn´s post hoc tests were carried out. Data from the performance assay was analyzed using a repeated measures ANOVA. To check whether phenolic compounds could explain the observed patterns of the performance assay, the analytes were one-by-one implemented as a co-variable into the repeated measurements model. Furthermore to compare survival of the caterpillars in the performance assay a Kaplan-Meier analysis was conducted.

Results

Emitter volatile emissions vastly increase by herbivore feeding

To evaluate the volatile signaling from emitter to receiver trees, volatile emissions from the emitting trees were collected. Altogether 27 volatile compounds could be identified (Table S1). Due to the herbivore treatment emissions of most volatile compounds increased drastically compared to levels constitutive emissions. Sesquiterpene emissions increased 4-fold, emission of aromatic compounds and monoterpenes increased about 8-fold, green leaf volatile emissions increased by 10-fold and *E*-DMNT emission increased by 65-fold. Nitrogenous compounds and isoamylacetate are exclusively emitted after herbivore feeding. A random forest analysis was run to elucidate the volatile compounds that differentiate the herbivore induced blend from constitutive emissions. Among the top ten differing compounds are the green leaf volatiles *Z-3*-hexenylacetate and *Z*-3-hexenol, the nitrogenous compounds *Z*-2-methylbutyraldoxime and *E/Z*-3-methylbutyraldoxime, the aromatic compound salicylaldehyde and the homoterpene *E*-DMNT (Table 1).

Phytochemical analyses in the receiver trees

Phytohormone levels are not affected by HIPV mediated signaling

To see whether HIPV signaling influences well-known plant hormones, salicylic acid, abscisic acid and jasmonic acid and its derivatives (referred to as jasmonates) were measured. All of the measured phytohormones showed a significant increase upon herbivore damage (Figure S1). The volatile signal, however, had no significant effect on any of the hormones.

Transcripts are not affected by HIPV mediated signaling

We used next generation sequencing to elucidate whether HIPV signaling is regulated at the transcriptional level. No annotated sequences were significantly expressed differentially comparing control leaves to HIPV exposed leaves (for data and more information see supplementary data).

Free sugars and total protein content are not affected by HIPV signaling

To check whether HIPV signaling has an effect on the nutritional value of a leaf, total protein content and free sugars were measured. The total protein content was not influenced by any treatment (Table 2). Similar the concentrations of sucrose, trisaccharides and tetrasaccharides were not significantly affected by any treatment (Table S4). In contrast glucose significantly increased after caterpillar feeding alone and fructose significantly increased upon herbivory (caterpillar treatment and HIPV exposed + caterpillar treatment) (Table S4). But, neither glucose nor fructose levels were influenced by HIPV signaling. Additionally we observed that the caterpillars dealt the same amount of damage to receiver trees that were exposed to constitutive black poplar emissions (caterpillar treatment: 7.49 ± 2.73 % leaf area loss) as to receivers that were exposed to HIPVs (HIPV exposed + caterpillar treatment: 8.36 ± 2.38 % leaf area loss; t-test showed no significant difference compared to caterpillar treatment).

Protease inhibitor content is not affected by HIPV mediated signaling

To test whether HIPV signaling influences the plants protease inhibitors, the level of all trypsin inhibitors was determined. Trypsin inhibitor concentration increased significantly upon herbivory but there is no additive (priming) effect upon previos HIPV exposure (Table S2).

Phenylacetaldoxime concentration is not affected by HPIV mediated signaling

Phenylacetaldoxime was measured to elucidate whether HIPV signaling affects the leaf storage of said compound. Herbivory significantly induced phenylacetaldoxime but no additional volatile signaling effect was observed (Table S3).

Salicin is primed by HIPV exposure with subsequent herbivore feeding

To check whether HIPV signaling influences the salicinoids, salicin and salicortin concentrations were measured. Salicin significantly increases after exposure to HIPVs and subsequent caterpillar feeding (Figure 2A). There is also a trend of slightly higher salicin concentrations after HIPV exposure alone and

caterpillar feeding alone. This trend is also apparent for salicortin. There are slightly higher concentrations after HIPV exposure and caterpillar feeding alone and in combination (Figure 2B).

Catechin is not affected by HIPV mediated signaling

Catechin was measured to see whether it was affected by HIPV exposure. None of the treatments had a significant effect on catechin (Figure 2C). However, there is a trend of lower catechin levels after exposure to HIPVs without subsequent feeding.

Caterpillars avoid HIPV exposed leaf discs

A caterpillar choice assay was performed to elucidate whether *L. dispar* caterpillars can distinguish between leaf tissue that was exposed to HIPVs and control leaves. Second instar *L. dispar* caterpillars fed significantly more on control leaf discs than on leaf discs that were exposed to HIPVs (Figure 3B).

Caterpillars performed worse when feeding on HIPV exposed leaves

To test whether feeding on HIPV exposed leaves affects *L. dispar* caterpillars, a performance assay was conducted. Caterpillars that were forced to feed on HIPV exposed leaves grew significantly slower compared to caterpillars feeding on control leaves (Figure 3A). Additionally a higher mortality was observed for caterpillar groups feeding on HIPV exposed leaves. Of all caterpillars of the respective group 9.41 % died in control treatment whereas 23.81 % caterpillars died in groups that were forced to feed on HIPV exposed leaves. A log rank test was run to determine if there were differences in the survival distribution for caterpillar groups feeding on control or HIPV exposed leaves. The survival distributions were significantly different, $\chi^2(1) = 6.24$, $p = 0.012$. Furthermore occasional cases of cannibalism were noticed in groups that fed on HIPV exposed leaves (S. Lackner, personal observation).

Analyses of leaves originating from the performance assay

Salicinoids are primed by HIPV exposure and subsequent herbivore feeding

To monitor whether the previously observed patterns can be found again in a different black poplar genotype, salicin and salicortin were measured in the leaves of performance assay after caterpillars had been feeding on them. Salicin and salicortin both increase significantly after HIPV exposure and subsequent herbivore feeding at time point 5 (Figure 4A+B). Furthermore, there is a trend of an increase of salicin upon HIPV exposure and herbivory at time points 3 and 8 (Figure 4A) and trend of an increase of salicortin at time point 3 (Figure 4B).

Catechin decreases upon HIPV exposure and subsequent herbivory

To check whether the previously observed trend can be found again in a different black poplar genotype, catechin was measured in the leaves originating from the performance assay. At harvest time point 3 catechin levels are not influenced by the treatment. However, at the two later time points catechin concentrations decrease significantly after HIPV exposure (Figure 4C).

Field data suggests that there is a negative correlation between salicortin concentration and L. dispar larval weight

In a common garden setting *L. dispar* caterpillars were reared on nine black poplar genotypes, which vary naturally in salicinoid content, to monitor the effect of different levels of salicinoids on larval development. Therefore salicin, salicortin and catechin were measured after the caterpillars fed on the trees for 14 days and larval weight was recorded. The salicin levels varied from a minimum of 1.66 mg g^{-1} DW to a maximum of 6.50 mg g^{-1} DW and salicortin levels varied from a minimum of 32.74 mg g^{-1} DW to a maximum of 115 mg g^{-1} DW. We found a significant logarithmic connection between salicortin and average caterpillar weight ($y = 0.342 – 0.06 * \log(x)$, $F = 8.139$, $P < 0.05$, $R^2 = 0.546$, Figure 5). There was no connection between neither salicin nor catechin content and larval weight (data not shown).

Discussion

For this study we wanted to investigate whether there is interplant volatile mediated signaling in poplar. Here we show that L. dispar caterpillars avoided feeding on primed leaves and performed worse when forced to feed on primed tissue. Furthermore, the salicinoids are primed and might serve as an explanation for the caterpillar behavior.

Black poplars herbivore-induced volatile blend has a huge signaling potential

The emitter volatile emissions of all major groups (green leaf volatiles, monoterpenes, sesquiterpenes, aromatic and nitrogenous compounds) highly increased after herbivory. Nitrogenous compounds were even completely absent on constitutive emissions (Table S1). It is well known that volatiles are inducible through herbivory. Green leaf volatiles are universally induced upon herbivore damage, there are numerous reports for herbaceous plants (Kigathi et al., 2009; Aharoni et al., 2003; Allmann and Baldwin, 2010) as well as for perennial species (Schmidt et al., 2011; Gossner et al., 2014; Arimura et al., 2004; Clavijo Mccormick et al., 2014; Frost et al., 2007). Additionally several monoterpenes, sesquiterpenes,

DMNT, aromatic and nitrogenous compounds are reported to be typically induced by herbivory (Arimura et al., 2004; Clavijo Mccormick et al., 2014; Danner et al. 2011).

Priming leads to higher mortality and lower performance of L. dispar caterpillars

We conducted bioassays with L. dispar caterpillars to elucidate whether HIPV exposed leaves would influence the caterpillars' behavior. In a choice assay the caterpillars fed significantly more leaf area of the control leaves (Figure 3 B). Apart from test bites the larvae avoided leaves that were previously exposed to HIPVs. So it is very likely that they perceived a gustatory signal. Since the fed on HIPV exposed leaf disc resembles the HIPV exposed + caterpillar treatment we hypothesize that salicin, which was the only primed compound we could measure, might be responsible for the feeding decision as it has been reported to be repellent to caterpillars before (reviewed in Boeckler et al., 2011). We also conducted a performance assay to check whether the observed patterns of the food choice assay has an impact on larval fitness. Significantly higher mortality was observed for caterpillars feeding on HIPV exposed leaves as well as significantly reduced larval weight gain (see results section and Figure 3 A). These findings lead to the conclusion that the poplar is better defended due to HIPV exposure. It has been reported that tobacco plants are more resistant to herbivores after receiving volatiles from a mechanically wounded sage bush (Karban et al., 2003). The increased mortality was most likely due to cannibalism. L. dispar is known to show cannibalism in populations with high density or when their nutrition is of poor quality (J. Mason et al., 2014). Since the density is constant during the performance assay it is very likely that the nutritional quality of the leaves decreased due to HIPV exposure. However, we could not measure any changes in free sugar concentration or total protein content (Table S2 + S4). Therefore the change in food quality could very well arise from increased defensive compounds like the salicinoids. We saw a priming of salicin (Figure 2) but the increase is very marginal and the abundance of salicin is in general very low compared to the abundance of salicortin which was not affected by HIPV exposure.

The leafs chemistry is mostly unaffected by priming

All of the measured phytohormones, namely SA, ABA, JA and its conjugates (referred to as jasmonates), were induced upon herbivory (Figure S1) but no effect was observed when the trees were exposed to HIPVs. It is well known that jasmonates are inducible through herbivory (reviewed by Wasternack and Hause, 2013). Furthermore it has been shown that ABA increases after herbivory as it is an important regulator for herbivore induced resistance via JA dependent defenses (Vos et al., 2013). SA on the other

hand is most often thought to play an important role in plant pathogen defense (Dempsey et al., 1999) but it has also been shown that SA is inducible upon sucking (Moran and Thompson, 2001) and chewing insects (Bi et al., 1997). However since none of the hormones reacted to HIPV exposure it is very likely that priming in black poplar is not regulated by SA, ABA or the jasmonates. Another possible way of regulating priming is a modification on the transcriptome level therefore we analyzed our samples with next-generation sequencing. We hypothesized that transcriptional changes, if applicable, should be visible after HIPV exposure but before the subsequent herbivore attack, thereby functioning as the memory for the initial herbivore attack, the priming stimulus. Therefore we compared the RPKMs of control and HIPV exposed treatment for all sequences but no significant differential regulation was found for an annotated sequence (supplementary material). There was also no difference between caterpillar and HIPV exposed + caterpillar treatment (data not presented). However if there were a difference between said two treatments it would much rather hint at enhanced gene expression by chromatin modification which is very common in systemic acquired resistance against pathogens (van den Burg and Takken, 2009; reviewed in Conrath, 2011). Now one could make the argument that the low replicate number might be the reason that we cannot find a pattern but we are convinced the method worked because we can find statistically significant differences between control and caterpillar treatment for sequences that are related to wounding and/or defense via JA (Figure S2). Therefore we conclude that in black poplar priming is regulated on a different level. There are numerous possible signal transduction ways like changes in cytosolic calcium, tricarboxylic acids, reactive oxygen species, membrane depolarization, hormone conjugates, amino acids, sugars or post transcriptional modifications (Mauch-Mani et al., 2017).

In Addition we checked whether HIPV exposure would influence levels of free sugars or total protein content, since as mentioned before they are possible signal transduction ways (Mauch-Mani et al., 2017) and would maybe alter the nutritional quality of a leaf from an herbivores perspective. However the total protein content was not influenced by any of our treatments (Table S2). As for the free sugars, glucose and fructose significantly increases after caterpillar feeding but again there is no effect of HIPV exposure (Table S4). We therefore concluded that the nutritional value of the poplar leaf does not change due to volatile priming. In addition to phytohormones and the transcripts, exposure to HIPVs did also not influence well-known defense compounds. We measured protease inhibitor concentrations since it has been proposed that PIs are affected by volatile defense priming (Farag et al., 2005; Kessler et al., 2006) but this is not the case in black poplar. We observed a significant induction of PIs after

herbivory but there is no significant effect of HIPV exposure (Table S2). We also measured phenylacetaldoxime, a semi volatile which accumulates in poplar leaves upon herbivory, that decreases the performance of L. dispar caterpillars (Irmisch et al., 2013). Furthermore it is a precursor of benzyl cyanide and other HIPVs (Irmisch et al., 2015). There was a significant induction of phenylacetaldoxime upon herbivory but again there is no significant influence of HIPV exposure (Table S3). We measured catechin, a precursor on condensed tannin biosynthesis that is connected to salicinoid biosynthesis as well. It is known that an overexpression of the condensed tannins pathway leads to reduced concentrations of salicinoids (Mellway et al., 2009; Boeckler et al., 2014). Catechin and the salicinoids therefore are believed to behave antagonistically. We observed a trend of decreased catechin levels in HIPV exposed leaves but none of the treatments caused a significant change to catechin levels (Figure 2). Additionally we measured catechin in leaves originating from the performance assay to confirm our results from the initial experiment. Interestingly catechin levels decreased significantly after six and nine days post HIPV exposure (Figure 4C). Now this decrease could be explained by the previously mentioned antagonism of salicinoid and condensed tannins biosynthesis pathways but it is also possible that HIPV exposure leads to an increase substrate turnover of catechin and therefore an increase of condensed tannins. However the effects of condensed tannins on herbivores remain unclear (reviewed in Barbehenn and Peter Constable, 2011). It has been shown in poplar that neither catechin itself nor condensed tannins have a negative effect on L. dispar caterpillars (Boeckler et al., 2014).

HIPV exposure primes salicinoid levels

Salicinoids represent another classic defense of the Salicaceae. They are reported to be toxic and deterrent to herbivores (reviewed in Boeckler et al., 2011). We measured salicin and salicortin to see whether HIPV exposure would influence these compounds. There is no effect of any of the treatments on salicortin but there is a significant increase of salicin in leaves that were exposed to HIPVs and subsequently attack by caterpillars. The literature shows no universal pattern whether salicinoids are constitutive and/or inducible defenses (Boeckler et al., 2011), which makes the priming of salicin even more interesting. We measured salicin and salicortin in the leftover fed upon leaves originating from the performance assay to elucidate whether the concentrations would change over a longer time period than the ones monitored during the initial experiment. There is a significant increase of salicin and salicortin in the leaves that were harvested six days after HIPV exposure (Figure 4A+B). Additionally there is a trend of increased salicortin and salicin four days after HIPV exposure and salicin is still increased at nine days post exposure. These results confirm our findings from the initial experiment. In

black poplar salicin can be primed quite rapidly, one day after HIPV exposure, and shows increased levels until at least nine days post exposure. The effect of priming on a more complex salicinoid is visible only after couple of days after the priming event and will vanish quicker compared to salicin.

Marginal increase of salicortin can influence caterpillar performance

So far to our knowledge HIPV exposure in poplar leads to significant though marginal increase in salicin and salicortin and to a decrease of catechin. However we see strong effects on caterpillar behavior and performance. The decrease in catechin cannot serve as an explanation for the performance reduction. On the other hand there are multiple reports of salicinoids being toxic to L. dispar (Boeckler et al., 2011) and that feeding caterpillars a diet with artificially enhanced amounts of salicin and salicortin results in slower growth and pupation rates (Hemming and Lindroth, 1995; Kelly and Curry, 1991; Orians et al., 1997). Therefore we performed a repeated measurements ANCOVA to check whether salicin or salicortin can explain the pattern of the performance assay. Indeed as a co-variable salicin has a significant impact on the weight gain of the caterpillars (AIC of the whole model 486.04; F-value of the effect of the co-variable salicin = 6.61, $p = 0.013$). Additionally we observed a significant negative correlation between increasing in salicortin concentration and larval weight (Figure 5) during a performance experiment that was set up in a common garden setting with natural varying concentrations of salicinoids between genotypes. Altogether, our findings strongly suggest that marginal increase of salicin and salicortin is indeed responsible for the decreased performance of L. dispar when feeding on primed leaves.

Conclusion

Though the actual mechanism of volatile-mediated defense priming still remains unknown our results suggest that exposure to HIPVs can prime salicinoids in black poplar trees for increased plant fitness upon subsequent herbivore attack. This supports that priming is an important mechanism for inter- and possibly intra-plant signaling (Frost et al., 2007; Li and Blande, 2017) in trees. Future studies will have to elucidate the actual signaling volatile compound and the underlying mechanism.

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Tables

Table 1: Top ten results of random forest analysis between constitutive or herbivore-induced volatile emissions from emitter trees. (MDA = MeanDecreaseAccuracy).

	MeanDecreaseAccuracy
E-β-ocimene	0.02502
Z-2-methylbutyraldoxime	0.0229
salicylaldehyde	0.021107
E-DMNT	0.02106
Z-3-hexenylacetate	0.020527
*E/*Z-3-methylbutyraldoxime	0.0204
Z-ocimene	0.020077
β-caryophyllene	0.018627
Z-3-hexenol	0.0184
isoamylacetate	0.01836

Figures

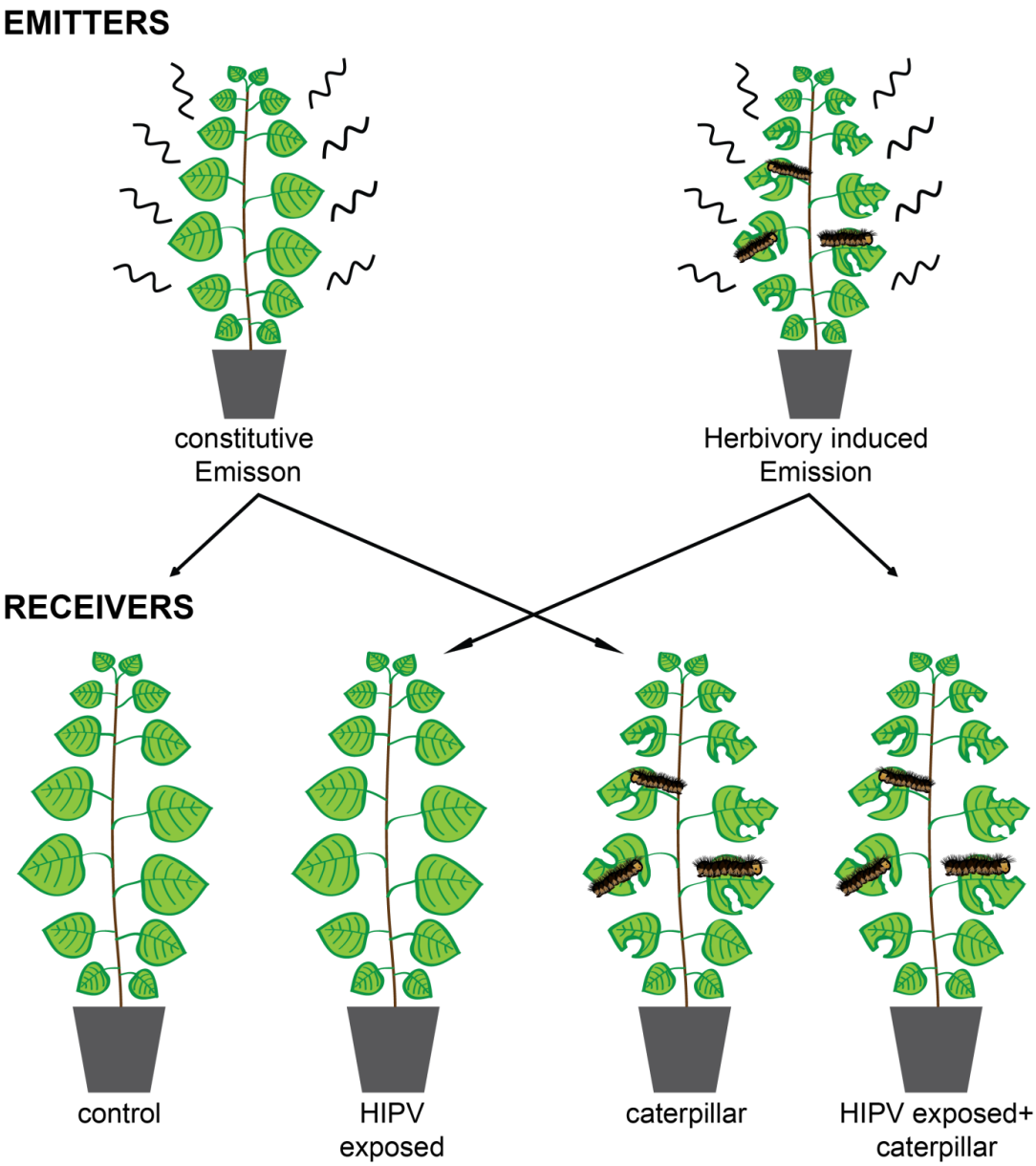

Figure 1: Experimental setup to prime the receiver trees. **"**Herbivory induced emission" emitter trees were infested with 10 4th instar *L.dispar* caterpillars. Directly after caterpillar onset, air connection was established for 48 h between emitter and receiver trees with an airflow of 0.5 l min^{-1}. Afterwards air connection was removed and receiver trees of the caterpillar and the HIPV exposed + caterpillar treatment were infested with 10 4th instar *L. dispar* caterpillars, which were allowed to feed an additional 24 h. Control and HIPV exposed trees rested an additional 24 h, leading to a total experimental time of 72 h. Emitter volatile emissions were collected for 2 h after the first 24 h of established air connection.

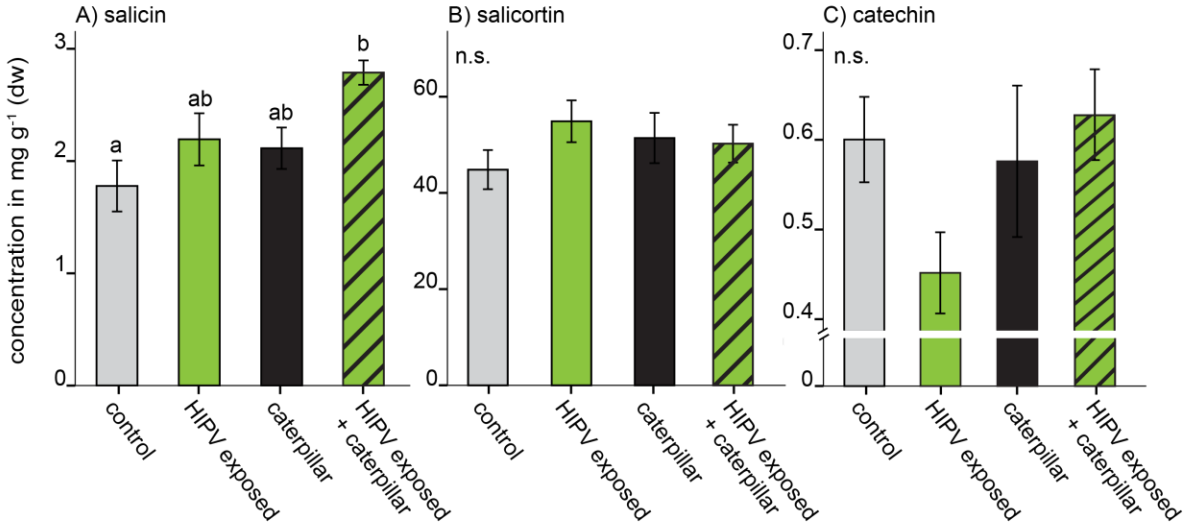

Figure 2: Salicin (A), Salicortin (B) and Catechin (C) concentrations of black poplar leaves after herbivore damage by gypsy moth caterpillars (caterpillar), previous exposure to HIPVs (HIPV exposed) and a combination of herbivore damaged and previously exposed to HIPVs (HIPV exposed + caterpillar) compared to control plants (control). Different letters indicate significant differences between treatments based on an ANOVA with Tukey´post hoc (A: F = 4.782; p = 0.012; C: n.s.) or a Kruskal Wallis test (B: n.s.). Bars represent means ± SE; n = 5-6.

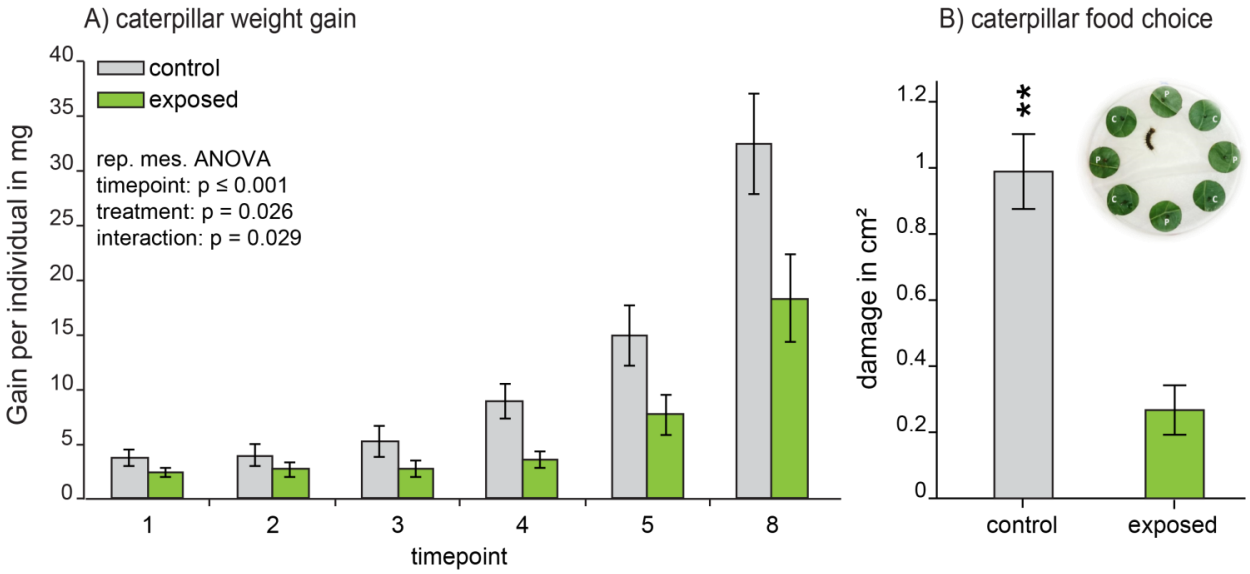

Figure 3: A: Caterpillar performance**.** Groups of 7 second instar *L.dispar* larvae were kept in separate petri dishes and forced to feed on either control or HIPV exposed leaves and weighed for 10 days. Dead larvae, if traceable, were removed before weighing. Offered leaves were exchanged twice, see method section for details. Bars represent means + SE. Given p-values result from a repeated measures ANOVA, n = 12. B: Caterpillar food choice**.** Leaf discs of either control or HIPV exposed trees were offered to one second instar *L.dispar* larva per petri dish arena. Caterpillars were allowed to feed for 24 h. Afterwards feeding damage was analyzed using Photoshop. Bars represent means +SE. Asterisks indicate significant difference based on a related samples Wilcoxon signed rank test: W= - 3.179, p = 0.001, n = 19.

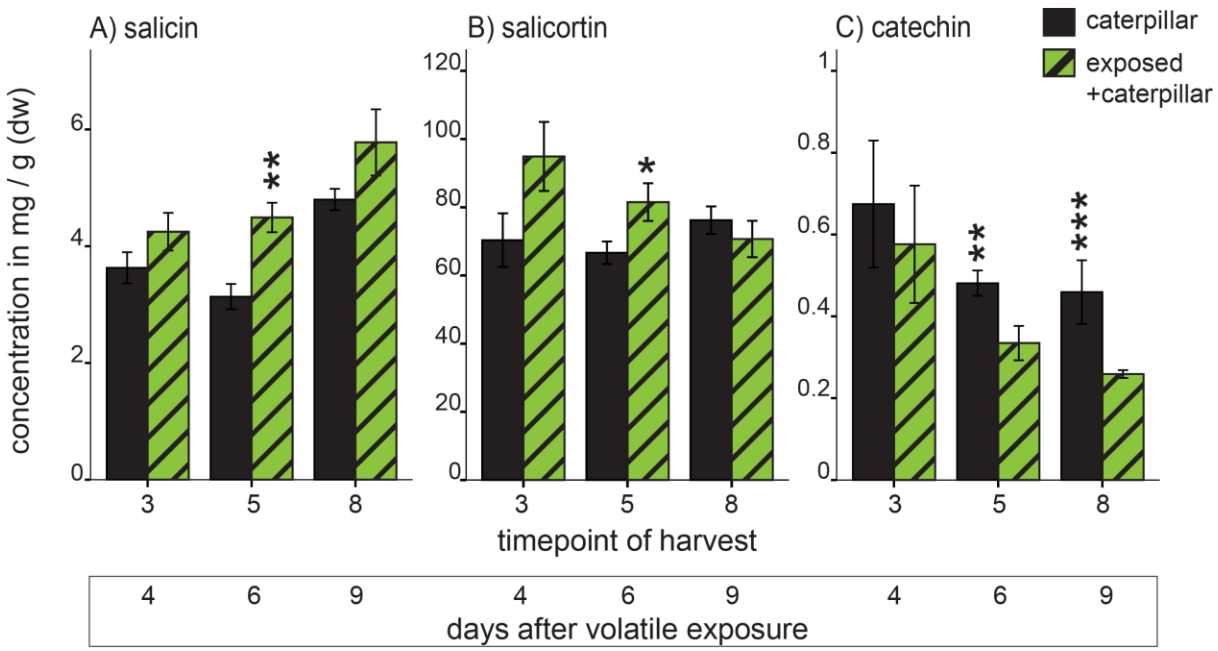

Figure 4: Salicin (A), Salicortin (B) and Catechin (C) concentrations of black poplar leaves analyzed after being fed on by caterpillars of the performance assay (Fig. 3A). Black bars represent leaves that were exposed to constitutive volatiles and afterwards offered to *L.dispar* caterpillars. Striped green bars represent leaves that were exposed to HIPVs and afterwards offered to *L.dispar* caterpillars. Displayed time points mirror time points of the performance assay (Fig. 3A). To compare differences between treatments within one time point a t-test was performed (A 5: $F = 18$, $p = 0.001$; B 5: $F = 37$, $p = 0.045$; C 5: $F = 23$, $p = 0.004$; C 8: $F = 10$, $p < 0.001$; all other comparisions were not significant) Asterisks indicate significance level based on the p-value. Bars represent means ± SE, $n = 12$.

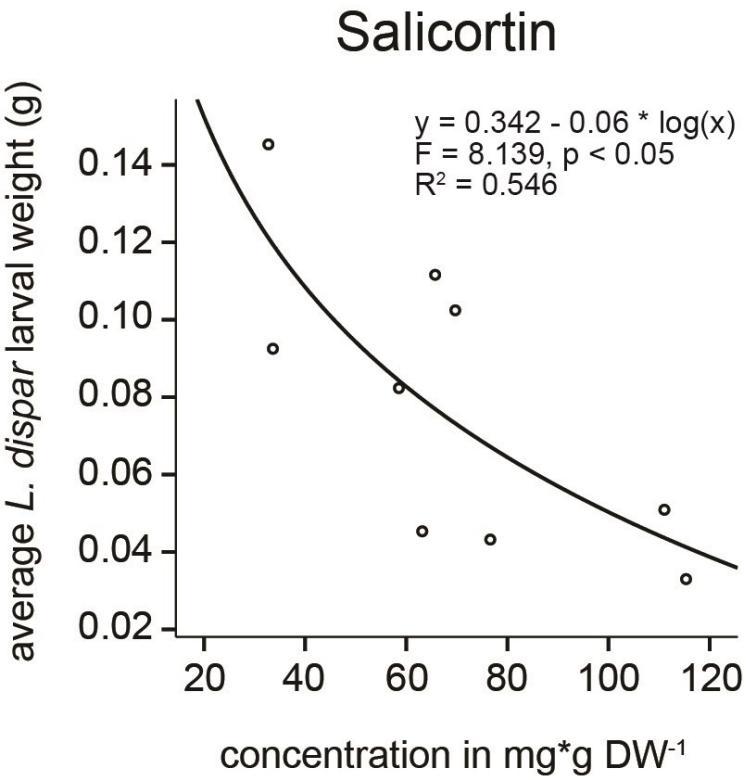

Figure 5: Average *L. dispar* larval weight in dependence of salicortin levels in black poplar trees of nine different genotypes. The data derives from a common garden experiment performed in July 2016. Twenty 2nd instar *L.dispar* caterpillars were allowed to feed on a leaf pool consisting of eight leaves for 14 days. Afterwards caterpillars were weighed (dead larvae excluded from analysis), leaves were harvested and analyzed for salicortin content. Shown are means of n = 5-10 larvae on each tree genotype (one sample per genotype).

Supplemental information

3.4 MANUSCRIPT IV

ONTOGENETIC DIFFERENCES IN BLACK POPLAR (POPULUS NIGRA) LEAF CHEMISTRY INFLUENCE FEEDING AND OVIPOSITION OF THE POPLAR LEAF BEETLE CHRYSOMELA POPULI

Thomas Fabisch, Jonathan Gershenzon & Sybille B. Unsicker

In preparation for *Journal of Chemical Ecology*

Formular 1

Manuskript Nr. 4

Titel des Manuskriptes: Ontogenetic differences in black poplar (*Populus nigra*) leaf chemistry influence feeding and oviposition of the poplar leaf beetle *Chrysomela populi*

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Bibliographische Informationen:

Der Kandidat / Die Kandidatin ist

☐ Erstautor/-in, ☐ Ko-Erstautor/-in, ☐ Korresp. Autor/-in, ☐ Koautor/-in.

Status: in Vorbereitung

Anteile (in %) der Autoren / der Autorinnen an der Publikation

Autor/-in	Konzeptionell	Datenanalyse	Experimentell	Verfassen des Manuskriptes	Bereitstellung von Material
Fabisch T.	70	100	100	80	-
Unsicker S.B.	15	0	0	10	-
Gershenzon J.	15	0	0	10	100

_____ _____

Unterschrift Kandidat/-in Unterschrift Betreuer/-in (Mitglied der Fakultät)

Title: Ontogenetic differences in black poplar (*Populus nigra*) leaf chemistry influence feeding and oviposition of the poplar leaf beetle *Chrysomela populi*

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Abstract

When searching for an optimal location for oviposition, gravid female herbivores typically orient to a mixture of relevant phytochemicals like free sugars, vitamins, lipids and amino acids but also secondary metabolites are influential. We investigated the potential influence of the ontogenetic distribution of some relevant compounds in black poplar (*Populus nigra*) trees on feeding and oviposition behavior of the specialized poplar leaf beetle *Chrysomela populi*. We also investigated how the decision where to oviposit affects offspring development as formulated in the "mother knows best" principle. We conducted feeding and oviposition assays *in planta* as well as *ex vivo* where beetles could choose between leaves of different developmental states, analyzed egg-induced changes and tracked the performance of the hatching larvae. Additionally, we analyzed black poplar metabolites possibly involved. In our study *C. populi* preferred younger leaves for feeding while at the same time avoiding these leaves for oviposition. The feeding behavior was reflected in the concentrations of free sugars and amino acids. The constitutive concentrations of salicinoids were not dependent on leaf age and therefore not influential, but interestingly, after one week of beetle infestation, the concentrations of salicortin and homaloside D increased exclusively in the upper leaves while salicin was not affected. The constitutive concentration of catechin was higher in aged leaves and might have accounted for the increased number of eggs found there. The simulation of egg deposition increased the levels of the phytohormone salicylic acid but the levels of jasmonic acid were very low and negligible. Egg simulation increased salicin concentrations exclusively in the slightly mature leaves while salicortin and homaloside D were not affected. The leaf age had no significant effects on larval weight gain and mortality, suggesting that larvae were not affected by any of the measured metabolic changes. Our results suggest that the decision of gravid *C. populi* females to oviposit on lower leaves had no influence on larval performance or mortality although differences in black poplar metabolites were observed. This shows the complexity of the relation between oviposition and larval performance in specialized insect herbivores.

INTRODUCTION

Insect herbivore species rarely perform parental care and thus gravid females should have sophisticated strategies to ensure the survival and fitness of their offspring. Finding an adequate spot for oviposition seems crucial in this context. The decision to choose a specific location for oviposition may be influenced by factors like the chemical preference, behavior and population structure of the insect as well as the distribution, quality and density of food plants (Singer 1971). An adult female may decide to oviposit on a specific host when a certain threshold of stimulant is reached (Jaenike 1990). This threshold is dynamic and influenced by the density of allo- and conspecifics, the presence of enemy-free space as well as the egg load and the search time available (Denno et al. 1990; Jaenike 1990). Additionally, female experience (Jones and Agrawal 2019; Ryan and Bidart-Bouzat 2014) and the level of feeding specialization (Cheng et al. 2013; Macel 2011) may affect the choice for an oviposition site although the latter is not a reliable argument since exceptions are known (Macel et al. 2002). In this context, host plant phytochemistry may play a very important role as a stimulant in oviposition. It is known, however, that plant phytochemistry can differently affect adult versus offspring performance (Garcia-Robledo and Horvitz 2012; Mason et al. 2019b). These differential effects present the basis for the question: do gravid females decide to oviposit on locations of greatest suitability for their offspring or rather for themselves? In the past, hypotheses were established, which tried to predict the optimal location for oviposition by gravid females. One well known hypothesis originally formed by Jaenike (1978) is the "oviposition-preference offspring-performance" hypothesis also known as "mother knows best" principle. According to this hypothesis females maximize their fitness by preferring locations for oviposition, which correspond to the best suitability for offspring development. However, studies testing this hypothesis found ambiguous results (Batallas et al. 2020; Garcia-Robledo and Horvitz 2012; Li and Liu 2015; Sun et al. 2020) and thus the mother knows best principle cannot explain all the oviposition patterns observed so far in the literature. Scheirs et al. (2000) showed that a female might also take the suitability for her own performance into account. Their hypothesis, known as the "optimal bad motherhood" hypothesis, is based on the assumption that females with maximized performance produce more eggs and therefore fitness increases. Both hypotheses are mainly applicable for the early stages of larval development where larval mobility is limited. During later stages larval mobility and consequently larval performance increases when larvae are able to move to the most suitable plant

parts (Galdino et al. 2015). The two hypotheses mentioned above show that the oviposition behavior in insects is a complex scenario.

Host plant chemistry is involved not only in host-finding but in feeding and many other aspects of herbivore life (Das et al. 2019; Kelly and Curry 1991). For example, it influences the whole reproduction process, including mating, oogenesis, the quality of the eggs and the development of the embryo (Hilker and Meiners 2011; Nishida 2014). However, herbivores are not able to perceive all secondary metabolites or all essential nutrients required but rather sense certain compounds, which act as stimulants or deterrents (Chapman 2003). Known phagostimulants are free sugars, free amino acids, lipids and vitamins (Chapman 2003; Hsiao and Fraenkel 1968; Maher et al. 2006; Matsuda 1988) but also secondary metabolites, the condition of the leaf surface and volatile organic compounds are of great importance, and many of these substances also play a role in oviposition (Das et al. 2019; Li et al. 2017; Maher et al. 2006; Roininen et al. 1999). It is believed that herbivores orient towards mixtures rather than single compounds (Islam et al. 1997; Pentzold et al. 2017; Tebayashi et al. 1995).

As in other plants, resources in poplar trees are not equally distributed throughout an individual but vary in time and space. Ontogeny is an important factor shaping the within-plant mosaic of metabolite concentrations (Raupp and Denno 1983; Whitham 1981). Juvenile leaf tissues, for example, are often of increased nutritional value since they contain higher amounts of foliar protein, nitrogen and water. Such nutritional status is known to affect herbivore performance with regard to consumption and relative growth rate (Barbehenn et al. 2015; Hunter and Lechowicz 1992). On the other hand juvenile tissues also contain increased amounts of defense-associated phytochemicals. As members of the Salicaceae family, poplar trees contain salicinoids (Boeckler et al. 2011), which are usually non-inducible defense metabolites (Boeckler et al. 2013; Osier and Lindroth 2001) although slight inductions are possible, dependent on the type of the salicinoid (Fabisch et al. 2019). Their concentrations decrease during leaf maturation (Boeckler et al. 2011). Anti-herbivore effects of salicinoids were evidenced for generalist moth species as well as specialized moth and leaf beetle species (Feistel et al. 2017; Hemming and Lindroth 1995; Kelly and Curry 1991) although some herbivore species are less affected or even are attracted by them (Rank 1992; Roininen et al. 1999; Soetens et al. 1991). Hence, in the phytochemical mosaic of a tree, the herbivore specialization level is an important determinant for finding optimal locations for feeding and oviposition. In poplar, susceptibility to salicinoids causes generalist herbivores to feed less on leaf tissues with higher salicinoid concentrations. For example, the gypsy moth (*Lymantria dispar*), a common generalist herbivore on poplar trees, is susceptible to salicinoids

(Hemming and Lindroth 1995; Osier and Lindroth 2001) and feeds less on juvenile leaf tissues (Kleiner et al. 2003). It can be assumed that generalist herbivores try to avoid high salicinoid concentrations when searching for locations for oviposition but unfortunately poplar studies investigating this topic are scarce. However, the behavior of Salicaceae-specialized herbivores is different because their tolerance to salicinoids makes exploitation of nutrient-rich leaves possible without suffering the negative developmental consequences. Consequently, specialized herbivores like e.g. chrysomelid beetles can often be observed feeding on young leaves with comparably high concentrations of salicinoids. An example of such an herbivore occurring on poplar is the chrysomelid beetle *Chrysomela populi*, specialized on Salicaceae trees with a strong preference for poplar trees (Urban 2006). Unfortunately, while it was recently shown that sucrose is a feeding stimulant for *C. populi* (Pentzold et al. 2019), little is known about other compounds involved in oviposition of this species, although the sequestration and attraction of *C. populi* to the salicinoid salicin suggests that it might be involved (Pasteels et al. 1983). This raises expectations that this beetle both feeds and oviposits on young leaves as already shown for a closely related species (Bingaman and Hart 1992; Bingaman and Hart 1993). However, preliminary observations on black poplar (*Populus nigra*) trees showed that *C. populi* discriminated between locations for feeding and oviposition. While the beetles fed intensively on the young leaves, they seemed to avoid these locations for oviposition. These observations raise questions about advantages for the hatching larvae, in the light of the “mother knows best” principle.

The aim of this study was therefore to investigate the oviposition preference-offspring performance relations of the specialized herbivore chrysomelid species *C. populi* within young black poplar trees. We addressed how feeding and oviposition behavior as well as larval performance depends on leaf ontogeny and measured phytochemical parameters that might be involved. Additionally, we were interested in temporal phytochemical changes caused by the presence of *C. populi* on black poplar trees as well as changes caused by the presence of *C. populi* eggs.

MATERIAL AND METHODS

Plants and insects

P. nigra saplings were grown from stem cuttings of a single genotype growing in a common garden near Jena, Germany (50°57'36.657" N, 11°.31'12.294" E). Stem cuttings were planted in 2L pots containing a 1:1 sand/soil mixture (Klasmann-Deilmann, Geeste, Germany) and cultivated in a greenhouse under summer conditions (24 °C; 60 % relative humidity; 16 h/8 h, light/dark) . Young trees were regularly fertilized and watered. Due to frequent mildew infections in the glasshouse, the lower leaves of the trees were gently wiped with wet paper cloth.

Chrysomela populi leaf beetles were reared from eggs of an in-house laboratory culture. Larvae were kept at room temperature and fed with foliage from young trees of the same *P. nigra* genotype as described above. Approximately 6 weeks after hatching, adult beetles started reproducing and these fertile adults as well as their offspring were used for the experiments.

Experimental design

In all experiments with young black poplar trees, an "upper" and a "lower" leaf pool was determined by counting 15 leaves from the first fully developed leaf on the apex in a basal direction. Leaves 1-5 constituted the "upper leaf pool" and leaves 11-15 the "lower leaf pool". These terms will be followed throughout the manuscript. The two leaf pools represented leaves in different developmental states. While the leaves of the upper leaf pool had just finished leaf expansion, the leaves of the lower leaf pool were older and comparably tougher.

In planta feeding and oviposition experiment

To study the feeding and oviposition behavior of *C. populi*, 20 young black poplar trees were selected. The experiment was conducted in the greenhouse and by the start of the experiment the trees were approximately 5.5 months old and 150 cm tall. Ten of the 20 young trees received three pairs of

copulating adult *C. populi* beetles (three females and three males). The other 10 trees served as controls and leaves from the "upper" and "lower" leaf pools (five leaves in each) were harvested at the beginning of the experiment to determine the phytochemical profiles the leaf beetles encountered (Fig. 1) The 10 beetle-infested trees were checked daily for the presence of egg clutches and their position. After 7 days, the adult beetles were removed from the trees, the number of eggs in each clutch counted, and the feeding damage determined as described below. Leaves in the two different leaf pools were then harvested. Eggs were removed from leaves and remains of egg clutches were gently washed away with water and a soft cloth to minimize egg contamination of subsequent leaf analyses.

Adult beetle choice assays with leaf discs in petri-dish arenas

To investigate whether adult beetles discriminate between the "upper" and "lower" leaf pool while feeding, a choice assay with black poplar leaf discs in petri-dish arenas was performed. Leaf discs were cut from the "upper" and "lower" leaf pools of four young black poplar trees. From each leaf within a leaf pool 3, discs were cut using a cork borer (diameter 16 mm), resulting in 15 leaf discs per pool and tree (Fig. S1). All leaf discs of the two leaf pools were pinned in the arenas in an alternating manner, so that each arena contained one leaf disc from each leaf pool of each tree, resulting in 8 leaf discs per arena. Adult *C. populi* beetles were randomly picked from the culture and starved for 6 h prior to the experiment. One adult beetle was then released in each of the petri-dish arenas. The arenas were placed next to each other on a laboratory bench. All arenas were covered with white paper cloth and beetles were allowed to feed for 24 h from 10:00 am in the morning to 10:00 am the next morning. Afterwards the beetles were removed from the arenas and the leaf discs were photographed after spreading them out on a whiteboard containing a reference area, for quantification of experimental herbivory as described in Boeckler et al. (2013). Subsequently the leaf discs of each treatment in each arena were pooled and transferred into 2 mL safety lock reaction tubes (Eppendorf Vertrieb Deutschland GmbH, Wesseling-Berzdorf, Germany) and flash-frozen in liquid nitrogen. Samples were stored at -20 °C until further processing.

Adult beetle oviposition choice assays with leaves *in planta*

16 pairs of *P. nigra* trees (age ~5.5 month, height ~150 cm) were vertically aligned to each other in a way that one leaf of the upper leaf pool of one of the paired trees was at the same height as one of the leaves of the lower leaf pool from the other tree. Both leaves together were enclosed in an air permeable cellophane bag (Armin Zeller, Nachfolger Schütz &Co, Langenthal, Switzerland), which was attached to the stem of each of the two plants using cable binders. Additionally, a horizontally aligned small wooden stick (diameter 5 mm) connecting both leaves was enclosed into the bag to improve mobility for the beetles and simulate the presence of the stem (Fig. S1). At the beginning of the experiment a copulating pair of *C. populi* leaf beetles was released into each bag. Trees were checked daily and the presence and position of egg clutches was documented. Once two egg clutches were present in one pairing, the beetles were removed and the plant prepared for the larval performance experiment described in the section "*C. populi* larval performance experiment on young black poplar trees". After removal of the beetles the number of eggs in each clutch was counted to later calculate the average number of eggs on each leaf.

***C. populi* egg-treatment experiment on upper vs. lower leaves of black poplar**

To investigate the effect of oviposition on the leaf chemistry of *P. nigra,* 30 trees were used. At the start of the experiment the plants were ~5 months old and ~140 cm in height.

The upper and lower leaf pools were marked as described in the "Determination of black poplar leaf pools for the experiments" section. On 10 of the 30 plants the youngest leaf of the lower leaf pool was marked. On another 10 plants the youngest leaf of the upper leaf pool was marked. On the last 10 plants the youngest leaves of both the upper and the lower leaf pools were marked (Fig. S2).

One leaf of each tree was treated with 15 mg of crushed *C. populi* egg material as illustrated in (Fig. S2). The egg material was prepared by grinding frozen eggs (age approximately 1 day post oviposition) collected earlier to a powder on liquid nitrogen before aliquoting 15 mg material each into 2 mL safety lock reaction tubes (Eppendorf Vertrieb Deutschland GmbH, Wesseling-Berzdorf, Germany). The material was then allowed to thaw prior to its application. The 15 mg represented the equivalent of the weight of one egg clutch containing 41 eggs, the average egg number of six clutch replicates observed. The application was performed on a specific pre-defined leaf area (diameter 20 mm) using a brush.

Control trees were treated the same way but instead of egg material water was applied onto the leaf. The treated leaves were left for 3 days before harvesting the pre-defined leaf area below the applied egg. Prior to that the egg material was washed away with water and a soft cloth before the leaf sample was collected. The water- treated samples of the upper and lower leaves of the 10 control trees were taken from the same tree (1 young and 1 old leaf per tree) but the egg-treated leaf samples were always collected from different trees (1 lower leaf or 1 upper leaf per tree, respectively).

Larval performance experiment

Two days before the experiment the trees of the larval performance experiment were transferred into a climate chamber (20 °C/18 °C, day/night: 60 % relative humidity; 16 h/8 h, light/dark) to acclimatize 48 h before the start of the experiment.

To study the performance of *C. populi* larvae in relation to leaf age, 20 *P. nigra* trees were selected. At the start of the experiment the trees were approximately 6 months old and 170 cm high. The experiment was conducted under greenhouse conditions described in the "Plants and insects" section. For each tree, one of the two leaf pools ("upper leaf pool" and "lower leaf pool") was defined as described in the previous section. The trees used for this experiment were the same trees as in the *in planta* oviposition choice experiment with paired leaves. After oviposition of the female on one of the leaves within the leaf pool, the beetles were removed and the eggs were allowed to stay on the leaf until the larvae hatched. As a result of this preparation, ten of the 20 trees received *C. populi* eggs on its upper leaf pool and another 10 trees received *C. populi* eggs on one leaf of the lower leaf pool (Fig. S3). After larval hatching their number in each clutch was reduced to 15. The larvae were confined to the respective leaves using plastic cages as described in (Eberl et al. 2020). At the start of the experiment the 15 larvae of each clutch were weighed. The initial average weight of each larva was determined by weighing all 15 larvae together and dividing their total weight by their number. Once the initial leaf was almost consumed, the cages containing the larvae were transferred onto the next leaf in apical direction. When the transfer of the cage was necessary for one tree, the cages of all trees were transferred to the next leaf as well. Every four days, the weights of the larvae were measured similarly to the initial weight measures by determining the total larval weight and dividing it by the number of larvae alive. After transferring the larvae from the first leaf to the second, a representative leaf sample was collected to analyze the chemical profile that the very young larvae were exposed to.

Chemical analysis

Plant harvest and leaf damage quantification

Subsequently to harvest or bioassay, the leaves or leaf discs of each leaf pool were photographed after spreading them out on a white board containing a reference area. Due to difficulties in homogenously grinding them to a powder, the midribs were removed when whole leaf samples were taken. Afterwards the leaves were flash-frozen in liquid nitrogen and stored in 5 ml plastic vials (and 2 ml safety lock reaction tubes as described before, respectively). The leaf material was then lyophilized (ALPHA 1-4 LDplus, Christ, Germany). Subsequently the leaf material was ground to a powder by using a paint shaker (Scandex, Pforzheim, Germany) and 5 steel balls of different diameters (Ø 3 mm – 5 mm). The herbivore damage quantified as leaf area loss was determined by analyzing digital images of the leaves with Adobe Photoshop (Version 15.0.0, Adobe Systems Incorporated, San Francisco, USA) as described in Boeckler et al. (2013).

Extraction of phytohormones, salicinoids, flavonoids, sugars, amino acids and phenolic acids

Defense hormones, sugars, salicinoids, flavonoids, amino acids and phenolic acids were extracted in a single procedure from an aliquot of 10 mg lyophilized leaf material. The material was dissolved in 1 mL of pre-cooled methanol (MeOH) containing the phytohormone standards D_6-abscisic acid (Santa Cruz Biotechnology, Dallas, TX, USA; 40 ng ml^{-1}), D_4-salicylic acid (Santa Cruz Biotechnology; 40 ng ml^{-1}), D_6-jasmonic acid (HPC Standards GmbH, Cunnersdorf, Germany; 40 ng ml^{-1}) and ^{13}C-jasmonoyl-isoleucine (synthesis described in Kramell et al. (1988)), using ^{13}C-Ile, Sigma Aldrich; 8 ng ml^{-1}). Additionally, trifluoromethylcinnamic acid (10 ng/mL) and syringic acid (10 ng/mL) as well as phenyl-ß-glucopyranoside (0.8 mg/mL) were added as internal standards for the quantification of phenolic acids and salicinoids, respectively. The samples were shaken for 30 s with a paint shaker before centrifuging them at 2000 g for 3 min. Afterwards 400 µL of the supernatant were transferred into a new tube. Another 200 µL were removed for the analysis of salicinoids and flavonoids. The rest of the supernatant was carefully removed from the tubes and discarded. Subsequently 1 mL of fresh MeOH (without labeled standards) was added to the remaining pellet and the procedure (shaker and centrifuge) was repeated. Another 400 µL (and 200 µL for salicinoid/flavonoid analysis) of the supernatant were collected and combined with the supernatant of the first extraction step. Additionally, 50 µL of the supernatant were separated for amino acid analysis. The extracts were stored at -20 °C until further use.

Phytohormone and phenolic acid quantification

For phytohormones and phenolic acids, the 800 µL MeOH extracts were analyzed non-diluted using high performance liquid chromatography (Agilent 1100 Varian ELSD, Varian, USA) coupled to a mass spectrometer (API 5000 LC/MS/MS System, AB Sciex, Framingham, MA, USA). The separation of the analytes was realized by injecting 5 µL analyte onto a C18 column (XDB-C18, 50 x 4.6 mm x 1.8 µm, Agilent, Santa Clara, CA, USA) using a formic acid (0.05 % in MeOH)/acetonitrile gradient with a flow rate of 1.1 mL/min. The column oven temperature was set to 25 °C. Analytes were detected via multiple reaction monitoring (MRM) in negative ionization mode (ion spray -4500 eV at 700 °C for phenolic acids and 650 °C for phytohormones) as described in Vadassery et al. (2012). The acquired data were processed using Analyst 1.6.3. (AB Sciex, Framingham, MA, USA). Phytohormones were quantified relative to the peak area of their corresponding internal standards while phenolic acids were quantified by comparing the peak area of the analytes to the recovery-corrected peak areas of the standards trifluoromethylcinnamic acid and syringic acid, respectively.

Amino acid quantification

The 50 µL of extract for amino acid quantification were mixed with 450 µL of milli-Q-purified water containing 10 µg/mL of an algal amino acid mix uniformly labeled with ^{13}C and ^{15}N (Isotec, Miamisburg, USA) and subsequently measured via high performance liquid chromatography (Agilent 1100 Varian ELSD, Varian, USA) coupled to a mass spectrometer (QTRAP 6500 LC/MS/MS System, AB Sciex, Framingham, MA, USA). Chromatographic separation was performed by injecting 2 µL analyte onto a C18 column (XDB-C18, 50 x 4.6 mm x 1.8 µm, Agilent, Santa Clara, CA, USA) using a formic acid (0.05 % in milli-Q-water)/acetonitrile gradient with a flow rate of 1.1 mL/min. The column oven temperature was set to 20 °C. Analytes were detected via MRM in positive ionization mode (ion spray 5500 eV at 650 °C). Nebulizer- and heating gas pressure was set to 70 psi and the curtain gas pressure to 40 psi. The retention times and MRM settings used are shown in table S1. The acquired data was processed using Analyst 1.6.3. (AB Sciex, Framingham, MA, USA). Amino acids were identified and quantified on the basis of authentic labeled standards.

Salicinoid and flavonoid quantification

For salicinoid and flavonoid analysis, 400 µL of the original extract were diluted 1:1 with 400 µL milli-Q-purified water just before their measurement via high performance liquid chromatography. Chromatographic separation of the analytes was realized using a chromatographic column (EC 250 x

4.6 mm NUCLEODUR Sphinx RP, 5 µm, Macherey Nagel, Düren, Germany) connected to a precolumn (C18, 5 µm, 4x3 mm, Phenomenex, USA). The temperature of the column oven was set to 25 °C. For separation, two solvents (Milli-Q-water and acetonitrile) were used from which acetonitrile was driven in gradient mode with time/concentration (min/%) of: 0:00/0; 19:00/52; 19:10/100; 21:00/100; 21:10/14; 26:00/14. The injection volume was set to 20 µL and the flow rate to 1 mL/min. The signal was detected via photodiode array and evaporative light scattering detectors (Varian, Palo Alto, CA, USA) at a wavelength of 200 nm. With the settings described above, salicin eluted at a retention time of about 5.1 min, salicortin at about 10.2 min and homaloside D at about 15.2 min and the flavonoid catechin at about 7.5 min. The compounds were identified by comparing their retention times to those from authentic standards isolated from previous work (Boeckler et al. 2013) and quantified on the basis of comparing their peak areas to those of the internal standard phenyl-ß-glucopyranoside, corrected by the recovery rate.

Sugar quantification

For sugar analysis, 50 µL of the original extract were 1:10 diluted by adding 450 µL milli-Q-water. Analysis was performed by high performance liquid chromatography (Agilent 1200 Varian ELSD, Varian, USA) coupled to a mass spectrometer (API 3200 LC/MS/MS System, AB Sciex, Framingham, MA, USA). Chromatographic separation was realized by injecting 5 µL analyte onto a hydrophobic interaction liquid chromatography (HILIC) column (apHera NH_2 15 cm x 4.6 mm I.D. 5 µm, Supelco, Bellefonte, PA, USA) using a milli-Q-purified water/acetonitrile gradient with a flow rate of 1.0 mL/min. The column oven temperature was set to 20 °C. Analytes were detected via MRM in negative ionization mode (ion spray -4500 eV at 600 °C). More details are described in Madsen et al. (2015). The acquired data was processed using Anlayst 1.6.3. (AB Sciex, Framingham, MA, USA). Sugars were quantified using an external standard curve with a mixture of glucose, fructose and sucrose (Sigma-Aldrich, St. Louis, MO, USA) ranging from 0.312 µg/mL to 10 µg/mL.

Statistical analysis

Simple statistical analyses, such as the comparison of two means, were carried out using the program SPSS Statistics version 20.0 (IBM, New York, USA). When normal distribution, homoscedasticity and the absence of outliers was present, simple comparisons were made by using *Student's TTest*. If these assumptions were violated, the non-parametric *Mann-Whitney U test* was used instead. For simple comparisons of related samples the *Wilcoxon signed rank test* was chosen. Multiple comparisons of the

phytochemistry data of the *C. populi in planta* choice experiment were carried out with a *generalized linear mixed effect model* carrying out a nested design with the tree ID as a random factor and the beetle treatment as well as the leaf pool as fixed factors. The models used for the analysis of the artificial egg-induction experiment also contained the tree-ID as a random factor and leaf pool as a fixed factor, but instead of the beetle treatment the egg treatment was implemented as the second fixed factor. The mixed effect models were built with the software R (version 3.6.1) and Rstudio (version 1.2.5033) using the packages “nlme” and “lme4”. Pairwise comparisons between the groups of these models were performed using Tukey tests available through the package “multcomp”. The models were chosen by first testing the significance of the random effect “tree ID” and subsequent stepwise inclusion of the fixed factors starting with the beetle treatment, continuing with the leaf pool and finally testing the interaction of the beetle treatment and the leaf pool. The best fitting models were chosen by their Akaike information criterion (AIC) values, their Likelihood-ratio and their levels of significance. Since assumptions of normality and homoscedasticity of the residuals were not met and could not be rescued with data transformation, differences in the larval performance could not be tested using a *repeated measures ANOVA*. .Instead for each timepoint a *Mann-Whitney U test* was applied to test potential significant differences of the average larval weights between the two leaf pools.

RESULTS

C. populi beetles fed and oviposited in different leaf pools

We assessed the amount of herbivore damage and the number of eggs of *C. populi* leaf beetles deposited on upper and lower leaves of black poplar plants after they were able to move freely inside an area of 15 pairs of upper and lower leaves for 7 days. During that time *C. populi* adult beetles caused significantly more damage (measured as leaf area loss) to the upper leaf pool compared to the lower leaf pool (Wilcoxon signed rank test: P = 0.005, Fig. 2). The artificial arena experiment with petri-dishes confirmed these findings under more controlled conditions. There, the beetles consumed significantly more biomass from leaf discs of the upper leaf pool than the lower leaf pool (P = 0.023, Fig. 3).

While the beetles preferred the upper leaves for feeding, the average number of eggs oviposited by the adult females during the *in planta* experiment was significantly higher on the lower leaf pool (Wilcoxon signed rank test: P = 0.007, Fig. 2). Also in the oviposition arena choice experiment the average number of *C. populi* eggs was significantly higher on the lower leaves when compared to the average number of eggs found on the upper leaves (P = 0.025, Fig. 3). Additionally, of 16 adult females used in this experiment 11 oviposited their first clutch on a leaf of the lower leaf pool while 5 females oviposited their first clutch on a leaf of the upper leaf pool. Furthermore 11 of the 16 females oviposited a second clutch on the same type of leaf while 5 females chose the opposite leaf for the oviposition of a second clutch. Only 2 of the females ovipositing their first clutch on leaves of the upper pool also oviposited a second clutch on the same upper leaf while 9 females ovipositing their first clutch on the leaf of the lower leaf pool also oviposited a second clutch on the same lower leaf (data not shown). Taken together these results showed that adult *C. populi* leaf beetles choose upper leaves for feeding and lower leaves for oviposition.

Amino acids and sugar concentrations differed between the leaf pools and were affected by leaf beetle presence

Since sugar and amino acid concentrations may influence the observed feeding and oviposition behavior of adult *C. populi* leaf beetles, we measured the levels of the free sugars glucose, fructose and sucrose as well as the levels of 15 amino acids (specified in table S1) in response to *C. populi* infestation and in

undamaged control trees. In general, we found higher sugar and amino acid concentrations in the upper leaf pool compared to the lower leaf pool. The presence of *C. populi* reduced the sugar concentration in both leaf pools, but the amino acid levels were not or only very slightly affected (Fig. 4). The sugar levels were significantly affected by beetle treatment ($t = -4.474$, $df = 18$, $p < 0.001$), leaf pool ($t = 4.641$, $df = 18$, $p < 0.001$) and the interaction of beetle treatment and leaf pool ($t = -4.221$, $df = 18$, $p < 0.001$, table 1). Post-hoc pairwise comparisons revealed significant differences between control and *C. populi*-infested trees in both leaf pools (Tukey contrasts post-hoc test, upper: $p < 0.001$, lower: $p < 0.001$). Additionally, we found significant differences in the total sugar levels between the upper and the lower leaf pools of the control trees (Tukey contrasts post-hoc test: $p < 0.001$) while this was not true for the *C. populi*-infested trees (Fig. 4). The concentrations of the amino acids were not significantly influenced by the beetle treatment, but by the leaf pool ($t = 7.636$, $df = 19$, $p < 0.001$, table 1). Post-hoc pairwise comparisons revealed significant differences in total amino acid concentrations between the upper and lower leaf pools of the control trees (Tukey contrasts post-hoc test: $p < 0.001$). Also the upper and lower leaf pools of the *C. populi*-infested trees were significantly different (Tukey contrasts post-hoc test: $p < 0.001$). Taken together our results show the significant influence of the leaf pool on both groups of primary metabolites measured, while *C. populi* infestation only affected the total sugar levels but not the total amino acid levels. The levels of sugars and amino acids were generally less concentrated in the lower leaf pool, suggesting that reduced feeding on these leaves may have been driven by the availability of sugars or amino acids.

Only salicin concentrations differed between the leaf pools but all salicinoids were affected by leaf beetle presence

Feeding and oviposition of *C. populi* beetles might also be influenced by the defense chemistry present in the tree at the moment of the arrival as well as by changes after feeding damage. Therefore, we analyzed the three major salicinoids, salicin, salicortin and homaloside D, found in black poplar. The mixed effect models showed that the salicin was significantly influenced by the beetle treatment ($t = 2.34$, $df = 18$, $p = 0.031$) and the leaf pool ($t = 2.597$, $df = 19$, $p = 0.017$, table 1). Pairwise comparisons revealed a significant difference between the upper leaf pool of the *C. populi*-infested plants and the lower leaf pool of the control trees (Tukey contrasts post-hoc test: $p = 0.004$, Fig. 5). Salicortin was significantly affected by the beetle treatment ($t = 2.681$, $df = 18$, $p = 0.015$), but was not influenced by the leaf pool (table 1). Pairwise comparisons revealed significantly increased levels of salicortin in the

upper leaf pool after *C. populi* infestation (Tukey contrasts post-hoc test: p = 0.003), while the levels in the lower leaf pool remained unchanged (Fig. 5). Similar to salicortin, homaloside D was only significantly affected by the beetle treatment (t = 2.158, df = 18, p = 0.045, table 1) with significant pairwise comparisons only between the upper leaf pools of control trees and trees infested by *C. populi* (Tukey contrasts post-hoc test: p = 0.045, Fig. 5). The similarities in salicinoid concentrations between leaf pools at early time points in the experiment suggest that feeding and oviposition location are not affected by salicinoids. However, *C. populi* infestation significantly increased the concentration of salicortin and homaloside D, but not the simple salicinoid salicin. These inductions might have affected feeding and oviposition at a later time point of the experiment.

Catechin concentrations differed between the leaf pools but were not affected by leaf beetle presence

Since some studies have pointed towards a possible involvement of flavonoids in insect oviposition, we also analyzed the concentrations of the flavonoid catechin. The mixed effect models showed that catechin was significantly influenced by the beetle treatment (t = 2.509, df = 18, p = 0.022) and the leaf pool (t = -5.703, df = 19, p < 0.001, table 1). Pairwise comparisons revealed significantly higher levels in the lower vs. upper leaf pools of the control trees (Tukey contrasts post-hoc test: p < 0.001) and significantly higher levels in the lower vs. upper leaf pools of the beetle-infested trees (Tukey contrasts post-hoc test: p < 0.001). Although the model suggested a significant influence of beetle presence on catechin concentrations, post hoc tests revealed no significant inductions caused by *C. populi* for both leaf pools although all levels increased slightly (Fig. S6). The results suggest that differences in the leaf pools (higher concentrations of catechin in the lower leaf pool) might have played a role in oviposition by *C. populi* females.

Stress-related phytochemical responses to the application of *C. populi* egg material

Egg-inducible defenses can have important impact on growth of the larvae after hatching. Our *in planta* experiment could not disentangle whether the observed salicinoid inductions were caused by feeding or by oviposition events. Therefore, we investigated egg-induced changes in poplar salicinoid levels and the stress-related phytohormones JA and SA by artificial application of *C. populi* egg material onto poplar

leaves belonging to either the lower or upper leaf pool. We used this approach because all leaves with egg clutches observed during the *in planta* experiment showed signs of herbivory as well.

In general, the concentrations of salicin and homaloside D were significantly affected by the leaf pool but not by the *C. populi* egg treatment, while the salicortin concentrations were all similar to each other (Fig. 6). The mixed effect model revealed that the concentration of salicin was significantly influenced by the leaf pool ($t = -3.272$, $df = 6$, $p = 0.017$) while the egg treatment showed no significant influence although a strong trend was observed ($t = 2.030$, $df = 23$, $p = 0.054$, table 1). Pairwise comparisons revealed significantly higher concentrations of salicin in the egg-treated leaves of the lower leaf pool when compared with the upper leaf pool (Tukey contrasts post-hoc test: $p = 0.005$) and a strong trend towards higher concentrations when compared with the control leaves of the lower leaf pool (Tukey contrasts post-hoc test: $p = 0.051$, Fig. 6). Salicortin and homaloside D were significantly influenced by the leaf pool (salicortin: $t = -2.587$, $df = 6$, $p = 0.041$; homaloside D: $t = -9.389$, $df = 6$, $p < 0.001$) but not by the egg treatment (table 1). Pairwise comparisons revealed no significant differences within the salicortin concentrations, but homaloside D was significantly increased in the leaves of the lower leaf pool, independent of the egg treatment (Tukey contrasts post-hoc test, upper C vs. lower C: $p < 0.001$, lower egg vs. upper egg: $p < 0.001$, Fig. 6).

Since they are often involved in the early steps of anti-herbivore defenses in plants, we also measured the egg-induced responses of the two biotic stress-related phytohormones JA and SA. The mixed effect models revealed that the JA levels were significantly influenced by the egg treatment ($t = 2.672$, $df = 23$, $p = 0.014$) as well as the leaf pool ($t = 8.338$, $df = 6$, $p < 0.001$, Table 1). Pairwise comparisons revealed significantly higher levels in the control leaves of the upper leaf pool when compared to the control leaves of the lower leaf pool (Tukey contrasts post-hoc test: $p < 0.001$) and in the egg-treated leaves of the upper leaf pool when compared to the egg-treated leaves of the lower leaf pool (Tukey contrasts post-hoc test: $p < 0.001$, Fig. S8). Additionally, egg-treatment of the leaves of the upper leaf pool led to increased concentrations of JA when compared to the corresponding control leaves (Tukey contrasts post-hoc test: $p = 0.042$), while this was not true for comparisons within the lower leaves where JA concentrations were below the limit of detection independent of the treatment (Fig. S8).

While the concentrations of JA were generally low and the response to the application of *C. populi* egg material comparatively weak, SA responded strongly to the treatment (Fig. S8). The mixed effect models showed that SA was significantly influenced only by the egg treatment ($t = 5.031$, $df = 23$, $p < 0.001$, table 1). Pairwise comparisons revealed significantly increased SA concentrations in the egg-treated

leaves of the upper leaf pool ($p = 0.017$) and the lower leaf pool ($p < 0.001$, Fig. S8). Our results suggest that both phytohormones responded to the application of *C. populi* egg material but the response patterns of JA and SA were different. Additionally, SA responded to the egg treatment much more intensely than JA.

The choice of the leaf pool had no influence on the larval performance of *C. populi*

To test, if the preference of gravid *C. populi* females to oviposit on the lower leaf pool is reflected in an increased performance of the hatching larvae on these leaves, as stated by the oviposition preference-offspring performance hypothesis, we tracked the average larval weight gain in each leaf pool within 16 days after hatching. In general, the leaf pool had no influence on the larval development (Fig. 7). At none of the measured timepoints was a significant difference observed in the average larval weights between the two leaf pools. These results show that the preference of adult females to oviposit on the lower leaves was not reflected in performance advantages for the larvae.

DISCUSSION

In this study we investigated the feeding and oviposition behavior of the highly specialized leaf beetle species *C. populi* on black poplar trees. We were additionally interested in egg-induced changes in black poplar phytochemistry and wanted to investigate if the choice of the location for egg deposition influences the performance of the offspring. The choice of a suitable location to oviposit is important for the survival of the hatching larvae, since their mobility is limited at least during the early stages of larval development.

***C. populi* leaf beetles discriminated between locations for feeding and for oviposition**

Our study on young black poplar trees showed that adults of *C. populi* preferred upper over lower leaves for feeding, but adult females preferred lower over upper leaves for oviposition. Thus, oviposition occurred in locations that were distinct from feeding sites. The preference of *C. populi* for younger over older leaves for feeding was also reported in an earlier study on *C. populi* (Urban 2006) as well as a study

on a closely related chrysomelid beetle species (Bingaman and Hart 1993; Ikonen 2002; Wait et al. 2002). However, the observation that *C. populi* oviposition on black poplar takes place in another location than feeding is different from what was reported for other chrysomelid beetles where correlations between leaf consumption and the distribution of egg masses were found (Augustin et al. 1993; Bingaman and Hart 1992; King et al. 1998).It is very likely that the oviposition choice of adult *C. populi* females was influenced by the chemistry or morphology of its host, since the chrysomelid species we used in this study is a specialist on poplar trees. Competition with conspecifics might have played a role, however, since it impacts the chemistry of the plant as discussed below. For a gravid female deciding where to oviposit, the constitutive as well as herbivory- or egg-induced leaf chemistry is important, since it can have a large impact on the suitability of a spot for the offspring.

Role of black poplar constitutive metabolite levels on feeding and oviposition by *C. populi*

When herbivores first arrive on a host, the constitutive chemistry will be decisive in determining suitable locations for feeding and oviposition. When searching locations for feeding, herbivores are typically stimulated by compounds like free sugars, free amino acids, lipids and vitamins (Chapman 2003; Hsiao and Fraenkel 1968; Maher et al. 2006; Matsuda 1988). The stimulating activity of sucrose was recently shown for *C. populi* (Pentzold et al. 2019) and free amino acids are known to be advantageous for herbivores since they can be assimilated from the gut without the necessity of proteolysis (Showler 2014). Our results showed that free sugars and amino acids were more highly concentrated in the upper leaf pool (Fig. 4), which might have explained the increased feeding events there. However, the reduced concentrations of these metabolites in the lower leaf pool do not likely explain the increased number of eggs found there. Therefore, other factors like secondary metabolites may have played a role as well.

Although secondary metabolites usually have deterrent activities on generalist-feeding insects they can be feeding stimulants for specialists, especially when these specialists sequester the metabolites in their own defense (Chapman 2003). Chrysomelid beetles occurring on Salicaceae plants are attracted to salicinoids (Kolehmainen et al. 1995, Rank 1992, Smiley 1985), and *C. populi* larvae sequester the salicyl alcohol moiety of salicin, which they oxidize to form salicylaldehyde, a major component of their defensive secretions against natural enemies (Bodemann et al. 2012; Michalski et al. 2008; Pasteels et al. 1983). In our study the initial salicinoid levels were not significantly different between the upper and lower leaf pools at the beginning of an infestation. However, the first egg clutches were laid after one

day of the experiment and 24 h are enough time for changes to occur in salicinoid concentration (Clausen et al. 1989). Therefore, salicinoids might still have played a role for oviposition of *C. populi* on black poplar at a later stage of infestation.

We also analyzed the levels of the flavonoid catechin, since the oviposition-inducing abilities of this compound have been described before. Ueno et al. (1990) found the oviposition of the weevil *Callosobruchus chinensis* to be increased when stimulated by D-catechin purified from azuki bean *Vigna angularis* although commercially purchased D-catechin required higher doses to induce oviposition. However, a study on the cerambycid beetle *Monochamus alternatus* conducted with fractions of extract made from the inner bark of its host *Pinus densiflora* showed that neither the identified D-catechin nor single fractions alone, but rather the combined extracts showed significant oviposition stimulant activity (Islam et al. 1997). Flavonol glucosides and glycosylated catechins were also found to be involved in the oviposition of this beetle species as well (Sato et al. 1999; Tebayashi et al. 1995). Thus, mixtures are probably more important than single proanthocyanidins or flavonol glucosides for the stimulation of oviposition. The higher constitutive levels of catechin we found in the oviposition-preferred lower leaf pool during our *in planta* experiment (Fig S6) are consistent with the promotion of *C. populi* oviposition by this compound.

Role of induced black poplar metabolite levels on oviposition by *C. populi*

The constitutive levels of black poplar chemical compounds probably gave the female beetles clues about the suitability of the leaves for their offspring and might have influenced their decision to oviposit on the lower leaf pool. However, the chemical characteristics of the trees changed after infestation by conspecifics. Secondary metabolites especially were induced differently in the upper and lower leaves as a result of the differential intensity of conspecific herbivory coupled with the differences caused by egg laying. This differential induction of poplar metabolites might have played an important role in the decision where to oviposit at least for egg clutches deposited later in the experiment. Most of the beetles fed on the upper leaf pool and therefore removed significant amounts of tissue, not available for the hatching larvae. The higher herbivore damage in the upper leaves also led to chemical changes in these leaves that were not observed in the lower leaves. First, herbivory by *C. populi* lead to significant changes in the leaf water content. It is well known that herbivory leads to water loss (Nykänen and Koricheva 2004; Ostlie and Pedigo 1984; Peschiutta et al. 2016) and the increased leaf area loss in the

upper leaves of the *C. populi*-infested plants caused their water levels to significantly drop, while this was not the case in the lower leaves where herbivory was much lower (Fig S9). Second, black poplars salicinoids were induced differently in the two leaf pools (Fig 5). The levels of salicin increased about one third after *C. populi* infestation in the lower leaf pool, while no changes were observed in the upper leaf pool. In contrast, the concentrations of salicortin and homaloside D both significantly increased in the upper leaf pool, while the lower leaf pool was unaffected. The suitability of the leaves for the hatching larvae might be influenced by a mixture of high salicin levels, which they require for sequestration (Pasteels et al. 1983), and low levels of complex salicinoids induced by conspecific herbivory in the upper leaf pool, which might have a negative effect on *C. populi* larvae as shown for *P. vulgatissima* (Kelly and Curry 1991). The differential salicinoid induction observed in our study supports the idea of the repelling function of salicortin in the oviposition process of *C. populi*, since it was only inducible in the upper leaf pool, which was avoided for oviposition. Third, herbivory on the upper leaves led to a significant increase of Kunitz-type trypsin (KTI) protease inhibitor activity (supplemental figure S10). These increases were exclusively observed in the upper leaf pools, probably as a result of the increased amount of damage caused by the herbivores. KTIs are known to be inducible by herbivore damage and possess anti-herbivore activity (Ma et al. 2011, Major and Constabel 2008, Phillipe et al. 2009). The exclusive induction of KTIs by intense conspecific herbivory in the upper leaves might have led to avoidance behavior of *C. populi* females, causing them to oviposit on the lower leaves instead. Future studies could investigate if KTIs have negative effects on the performance of *C. populi* larvae.

Egg-induced changes in black poplar

We investigated the phytochemical response of black poplar to *C. populi* eggs by applying egg material onto a specific area of leaves from the upper or lower leaf pools. Of all three measured salicinoids, only salicin responded and it was increased exclusively in the lower leaves. Both complex salicinoids were not affected. Differences in induction patterns of salicinoids have already been described before (Fabisch et al. 2019; Fields and Orians 2006) and probably arise because of the different biochemical pathways involved in the biosynthesis of salicin compared to the more complex salicinoids (Babst et al. 2010). Such differential induction might indicate different ecological functions, and our data suggest that salicin is involved in egg-induced defenses in black poplar while the complex salicinoids are not. Besides salicin, none of the other measured black poplar primary and secondary metabolites responded to *C. populi* egg treatment. However, some changes in the phytohormone levels were observed. Oviposition of insect

eggs is known to influence the phytohormone levels of the host plant. Past reviews have highlighted the involvement of JA and SA in egg-induced defenses (Bertea et al. 2020; Hilker and Fatouros 2015). In our study we measured both phytohormones but found only SA to be significantly triggered by *C. populi* eggs (Fig S8). JA was more concentrated in the upper leaves and slightly induced by egg treatment, but the concentrations were very low compared to the herbivory-induced levels observed in black poplar during past studies (Fabisch et al. 2019; Lackner et al. 2019) or during the in planta choice experiment in this study (Fig S4). The minor change of JA in response to the egg treatment suggests that SA is involved in egg-induced defense responses of black poplar, while JA has at most weak importance. However, during our *in planta* choice experiment the induction patterns of the phytohormones were somewhat different. SA was not induced by *C. populi* infestation, but showed higher concentration in the upper leaf pool of the control plants. In fact, the SA levels of the upper leaf pool were reduced in trees with *C. populi* infestation (Fig S4). One possible reason for this observation might have been the induction of herbivory-triggered JA in both leaf pools, which in turn antagonized SA levels. It is widely acknowledged that phytohormones such as SA and JA, undergo crosstalk (Glazebrook 2005; Yang et al. 2019). Our results suggest such crosstalk too and since all of the leaves with *C.populi* eggs also showed at least small traces of herbivory, the subsequent induction of JA might mediate against the egg-induced changes triggered by SA.

The choice of the location for oviposition did not affect *C. populi* larval performance

During our study we were also interested if any of the black poplar chemical changes observed influenced the performance of the larvae, which might justify the decision of gravid females to oviposit onto the lower leaf pool. Based on the changes in the salicinoid levels during the *in planta* feeding and oviposition experiment, especially the induction of salicortin in the upper leaf pool of *C. populi*-infested plants, we expected the performance of the *C. populi* larvae to be better on the lower leaf pool. Interestingly, this was not the case. All larvae performed equally independent of the leaf pool they fed on (Fig 7). This shows that salicinoids do not have the same negative influence on *C. populi* performance, as previously described for some generalist herbivore species (Hemming and Lindroth 1995; Lindroth and Peterson 1988; Osier and Lindroth 2001) and even the specialist herbivore species *P. vulgatissima* (Kelly and Curry 1991). The fact that *C. populi* larvae performed similarly on both leaf pools although differences in their metabolite profile were found raised a question about the changes in salicinoid levels during larval herbivory, but these were not different (Fig S7). It seems that after 8 days of larval

herbivory the concentrations of these defense metabolites in the leaves were similar in both leaf pools, independently of their levels before hatching. This does not necessarily mean that salicinoids play no role in larval development since differences might be present very early after hatching, which might influence the concentrations in larval defensive secretions and hence defense against predators and parasites. But, we did not assess the influence of *C. populi* feeding site on its ability to defend itself during this study. Future studies should focus on this question to better understand the role of salicinoids in larval development.

Evaluation of the oviposition preference-offspring performance principle in a within-plant setting

The oviposition preference-offspring performance principle states that gravid females oviposit on locations that optimize benefits for their offspring. Our investigations into the feeding and oviposition of the leaf beetle species *C. populi* on black poplar show that the oviposition preference-offspring performance principle is not applicable to this interaction. The choice of gravid females to oviposit on the lower leaves was not reflected in the performance of the offspring. Instead, all larvae performed equally, independent of the age of the leaves they consumed. However, our conclusions may be limited by our measures of performance. We measured larval weight gain, mortality and pupal weights, but in our greenhouse-based study we could not determine how well larvae feeding on different leaf pools were defended against predators and parasitoids or were fit for competition against con-and allospecifics. Taking these parameters into account might result in different conclusions.

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FIGURES

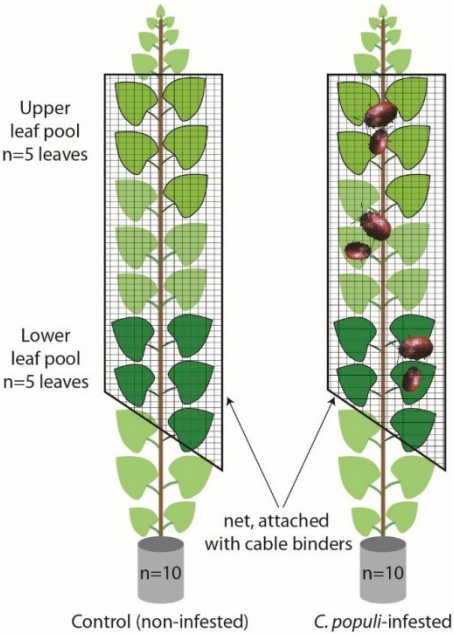

Fig1: Experimental scheme for the investigation of herbivory and oviposition behavior of the chrysomelid leaf beetle *C. populi* on young black poplar (*P. nigra*) trees.

Starting from the youngest fully developed leaf and counting in a basal direction 15 leaves of 20 young black poplar trees (age approximately 4 months, height approximately 150 cm) were enclosed in a net. The nets were attached to the tree using cable binders. Of these 20 trees, 10 trees each received 3 pairs of copulating *C. populi* leaf beetles, which were allowed to freely move within the net for 7 days, while a further 10 trees did not receive beetles and therefore served as control trees. Within the 15 leaves of each tree, 2 leaf pools consisting of 5 leaves each were marked visually. A pool consisting of the 5 youngest leaves was designated as the “upper leaf pool” and another pool consisting of the 5 oldest leaves was designated as the “lower leaf pool”. The control trees were harvested at the beginning of the experiment to reflect the situation on the plant when the beetle arrives. The beetle-infested trees were harvested at the end of the experiment to obtain information about changes in leaf chemistry caused by the presence of the leaf beetle. Every day during the 7 days of the experiment, all leaves of the beetle-infested trees were checked for the presence of *C. populi* eggs. If eggs were detected, the leaf number was noted. At the end of the experiment, the leaf area loss in each of the two leaf pools was determined digitally to investigate the extent of herbivory. Also the eggs of the clutches were counted and the average egg number was calculated for each tree and leaf pool to investigate the extent of oviposition events.

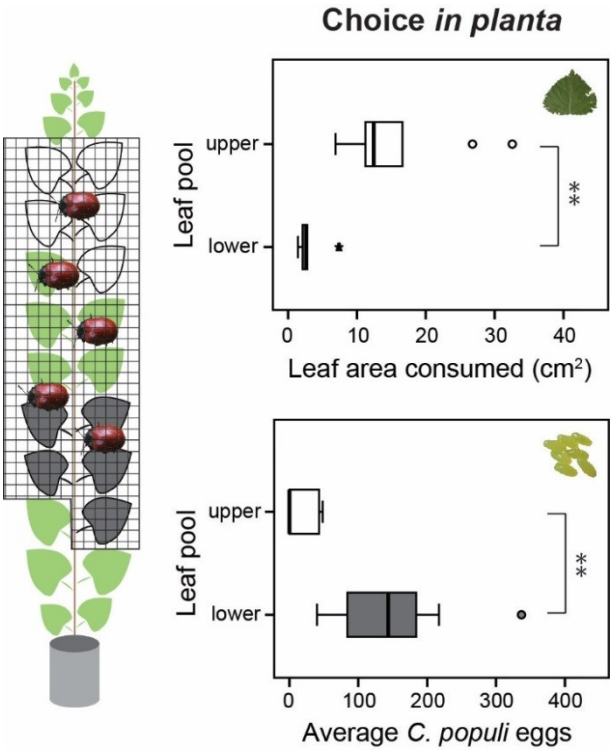

Fig2: Leaf pool preferences for oviposition and feeding events by the leaf beetle *C. populi* on young black poplar (*P. nigra*) trees tested *in planta*.

Shown is the extent of herbivore damage caused by 6 adults (3 males and 3 females) and the average egg number of 3 female chrysomelid *C. populi* leaf beetles per tree under natural conditions on black poplar trees in relation to the leaf pool (upper = white, lower = grey bars). The beetles were allowed to freely move within an area of 15 leaves starting from the youngest fully developed leaf and counting in a basal direction for 7 days before the leaf area loss and the number of eggs was determined. The damage caused by the adult beetles was significantly higher in the upper leaf pool (related samples Wilcoxon signed rank test, $p<0.01$) while significantly more eggs were found in the lower leaf pool (related samples Wilcoxon signed rank test, $p<0.01$). The boxes depict medians ± 1.5 interquartile range of n=10 plant replicates. The boxes represent the median ± 1.5 interquartile ranges. Circles represent outliers and 5-pointed stars represent extreme outliers. 6-pointed stars represent the level of significance for the tests depicted inside the boxes.

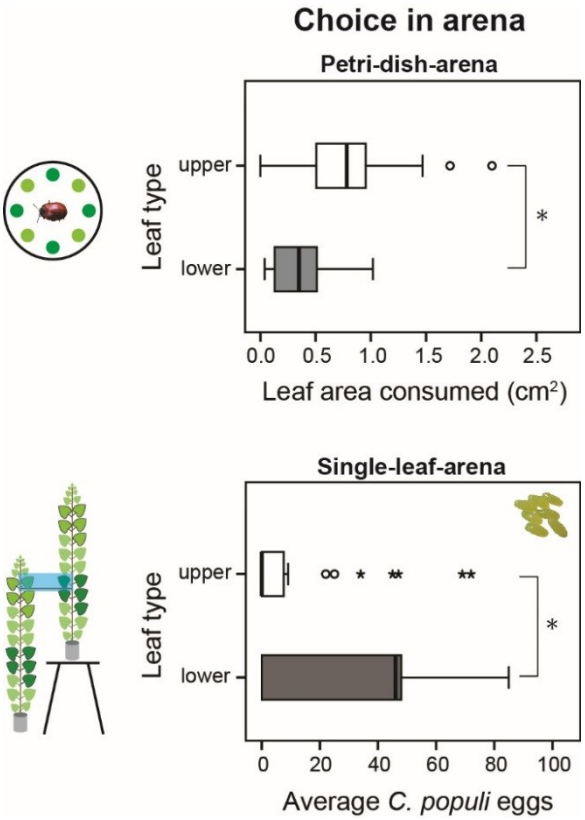

Fig3: Leaf pool preferences for oviposition and feeding by the leaf beetle *C. populi* on black poplar (*P. nigra*) leaves tested *ex vivo*.

The upper figure depicts the extent of herbivore damage on poplar leaf discs (diameter 16 mm) by adult beetles trapped into petri-dish-arenas for 24 h (n=15 arenas / 1 beetle per arena). Each arena contained 8 leaf discs, 4 from leaves of upper (white bars) and 4 from leaves of lower leaf pools (grey bars), which were pinned in there in an alternating manner. The lower figure depicts the average number of eggs found on upper (white bars) and lower (grey bars) poplar leaves, which were horizontally aligned to each other in a way that one leaf of the upper leaf pool of a tree was horizontally aligned to one leaf of the lower leaf pool of another tree. In each *in planta* arena one copulating pair of beetles was released, which could freely move between the leaves until two egg clutches were layed. Afterwards the number of eggs was documented for each leaf. A more detailed description of the experimental setup is depicted in Fig. S1. The damage caused by the adult beetles in the petri-dish-arenas was significantly higher on leaf discs of the upper leaf pool (paired samples T-test, $p<0.05$). In the *in planta* arena significantly more eggs were found in the lower leaf pool (related samples Wilcoxon signed rank test, $p<0.05$). The boxes depict medians ± 1.5 interquartile ranges. Circles represent outliers and 5-pointed stars represent extreme outliers. 6-pointed stars represent the level of significance for the tests depicted inside the boxes.

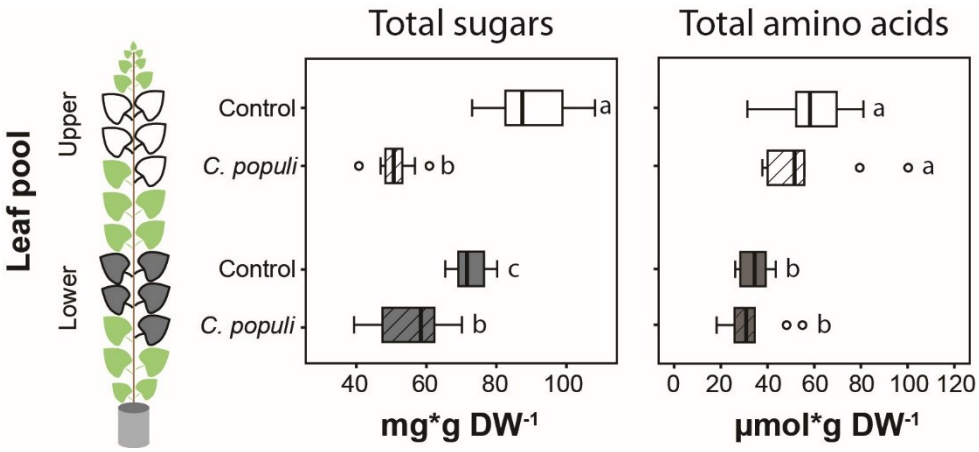

Fig4: Concentration of free sugars and amino acids in two different leaf pools of young black poplar (*P. nigra*) trees infested by the chrysomelid leaf beetle *C. populi* for 7 days and uninfested control trees.

Shown are the summed concentrations of the sugars glucose, fructose and sucrose as well as the summed concentrations of the amino acids Ala, Ser, Pro, Val, Thr, Ile, Leu, Asp, Glu, His, Phe, Tyr, Trp, Asn, Gln, and Lys in upper (white boxes) and lower (grey boxes) black poplar leaves (n=10) after one week of herbivory and oviposition by the specialized chrysomelid leaf beetle *C. populi* (dashed boxes) as compared to non-infested control trees (blank boxes). The control leaves (n=10) were harvested at the start of the experiment to reflect the situation the adult beetles were confronted with when they arrived at the plant. The boxes represent the median ± 1.5 interquartile ranges. Circles represent outliers. Small letters on the right side of the boxes represent the results of Tukey's post-hoc analysis performed subsequently to a mixed effect model. Results of this model are presented in table 1.

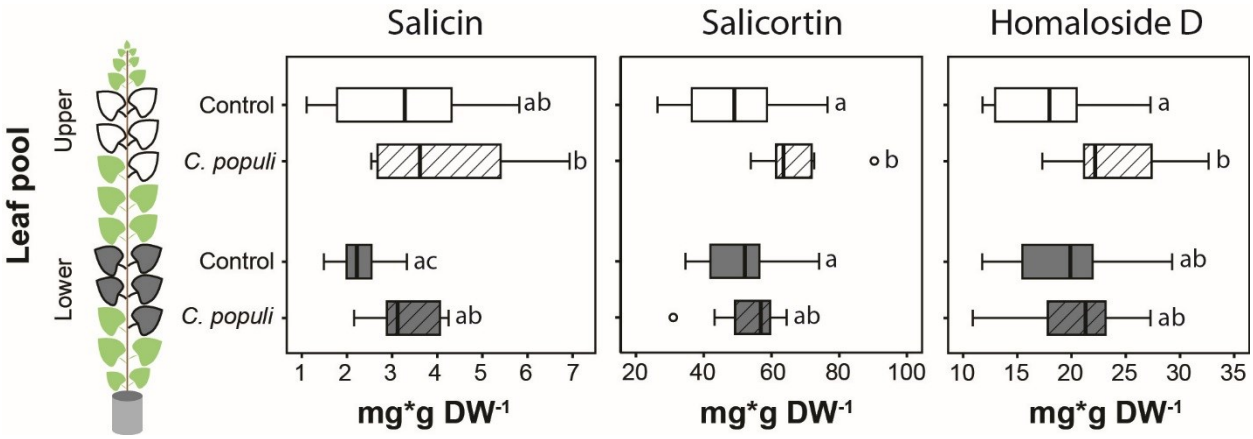

Fig5: Concentration of salicinoids in two different leaf pools of young black poplar (*P. nigra*) trees infested by the chrysomelid leaf beetle *C. populi* for 7 days and uninfested control trees

Shown are the concentrations of the salicinoids salicin, salicortin and homaloside D in upper (white boxes) and lower (grey boxes) black poplar leaves (n=10) after one week of herbivory and oviposition by the specialized chrysomelid leaf beetle *C. populi* (dashed boxes) as compared to non-infested control trees (blank boxes). The control leaves (n=10) were harvested at the start of the experiment to reflect the situation the adult beetles were confronted with when they arrived at the plant. The boxes represent the median ± 1.5 interquartile ranges. Circles represent outliers. Small letters to the right of the boxes refer to the results of a Tukey post-hoc analysis. Small letters on the right side of the boxes represent the results of Tukey's post-hoc analysis performed subsequent to a mixed effect model. Results of this model are presented in table 1.

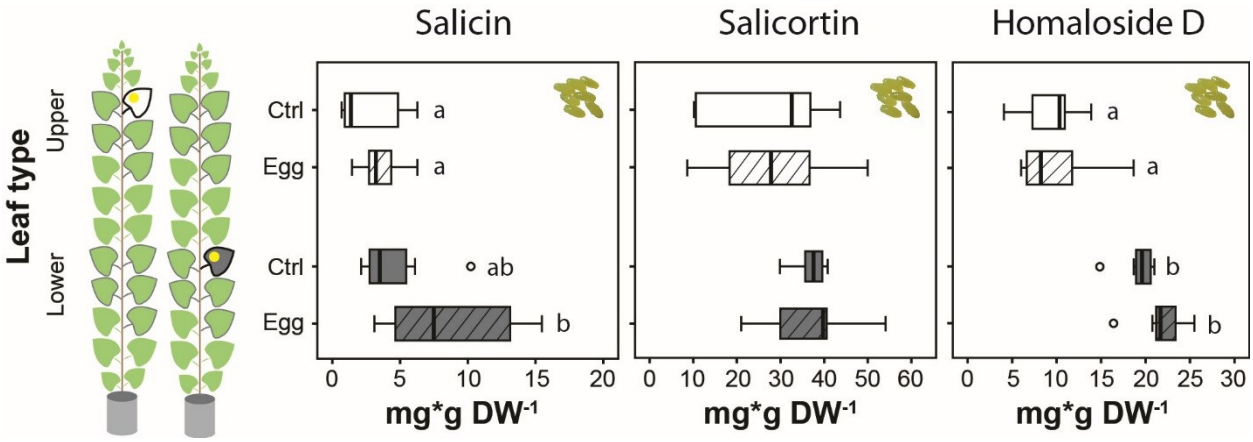

Fig 6: Induction of salicinoids by artificial application of crushed *C. populi* egg extracts

Depicted are the concentrations of the salicinoids salicin, salicortin and homaloside D in young black poplar trees three days after artificial application of crushed *C. populi* egg material (dashed boxes) on a pre-defined location (diameter 20 mm) of representative leaves belonging to the upper (white bars, control n=6, egg n=9) and the lower (grey bars, control n=8, egg n=9) leaf pool. The odd number of replicates resulted from the loss of some samples due to contamination during the processing. The material was applied using a soft brush. Control leaves (blank boxes) were treated with water instead. Shown are the concentrations of the leaf discs harvested directly beneath the application site. For application, a similar amount of egg material as an average clutch weight of *C. populi* was used. Before collecting the leaf sample, the egg material was removed gently with water and a soft cloth. The boxes represent the median ± 1.5 interquartile ranges. Circles represent outliers. Small letters on the right side of the boxes represent the results of Tukey's post-hoc analysis performed subsequent to a mixed effect model. Results of this model are presented in table 1.

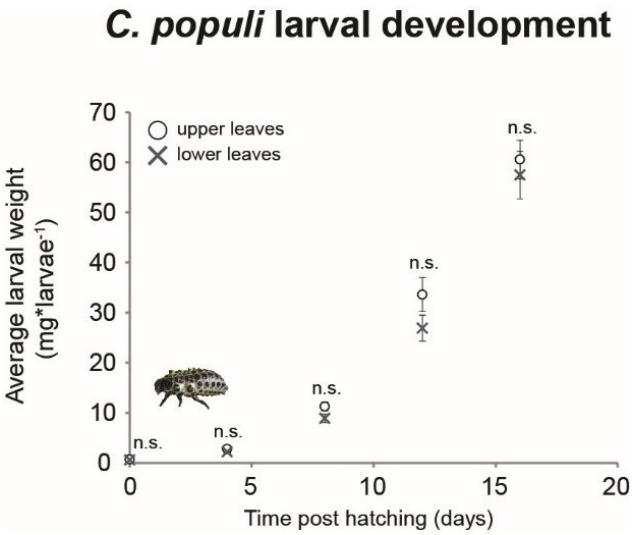

Fig7: Larval performance of *C. populi* on upper and lower leaves of young black poplar (*P. nigra*) trees.

Depicted is the performance of *C. populi* larvae on upper (dots) and lower (crosses) leaves of young black poplar trees. Every tree contained only one (upper or lower) leaf pool. The data points represent the average larval weights plus standard errors (black bars) of n=10 plant replicates with n=15 larvae per plant initially caged onto each leaf. The larvae originated from eggs, which were earlier oviposited onto the respective leaves by *C. populi* females. The number of 15 larvae was obtained by reducing the larvae in the clutch after they hatched. The average larval weight resulted from the total weight of all surviving larvae divided by their number. The larvae were weighed every 4 days until 16 days. Significance between the larval weights in the two leaf pools was tested for each time point using a Mann-Whitney U test.

Table 1 Results of the mixed effect models and their corresponding model summaries of phytochemical conditions in young *P. nigra* trees.

***in planta* experiment**						**treatment**			**leaf pool**			**treatment*leaf pool**		
Compound	**Model**	**df**	**AIC**	**L-Ratio**	**p**	**t**	**df**	**p**	**t**	**df**	**p**	**t**	**df**	**p**
Total sugars	Sugars ~ treatment * leaf pool	6	290.037	16.082	**<0.001**	-4.474	18	**<0.001**	4.642	18	**<0.001**	-4.221	18	**<0.001**
Total amino acids	Amino acids ~ treatment + leaf pool	5	878.094	28.918	**<0.001**	-0.368	18	0.718	7.636	19	**<0.001**	-	-	-
Salicin	Salicin ~ treatment + leaf pool	5	133.115	6.216	**0.013**	2.342	18	**0.031**	2.597	19	**0.018**	-	-	-
Salicortin	Salicortin ~ treatment	4	323.356	6.930	**0.009**	2.681	18	**0.015**	-	-	-	-	-	-
Homaloside D	Homaloside D ~ treatment	4	246.705	4.622	**0.032**	2.158	18	**0.045**	-	-	-	-	-	-
Catechin	Catechin ~ treatment + leaf pool	5	147.536	20.578	**<0.001**	2.509	18	**0.022**	-5.703	19	**<0.001**			
Jasmonic acid	Jasmonic acid ~ treatment	4	57.445	16.970	**<0.001**	5.038	18	**<0.001**	-	-	-	-	-	-
Salicylic acid	Salicylic acid ~ treatment * leaf pool	6	66.403	4.204	**0.040**	0.022	18	0.983	2.705	18	**0.015**	-1.997	18	0.061
Phenolic acids	Phenolic acids ~ treatment * leaf pool	6	252.929	8.428	**0.004**	0.438	18	0.666	11.601	18	**<0.001**	-3.071	18	**0.007**
Leaf water content	Water ~ treatment + leaf pool	5	186.628	14.191	**<0.001**	-2.362	18	**0.030**	-4.370	19	**<0.001**	-	-	-
egg-induction experiment														
Salicin	Salicin ~ treatment + leaf pool	5	170.313	10.05	**0.002**	2.031	23	0.054	-3.272	6	**0.017**	-	-	-
Salicortin	Salicortin ~ treatment + leaf pool	5	247.037	6.618	**0.010**	-0.043	23	0.966	-2.587	6	**0.041**	-	-	-
Homaloside D	Homaloside D ~ treatment + leaf pool	5	173.177	44.676	**<0.001**	1.505	23	0.146	-9.389	6	**<0.001**	-	-	-
Jasmonic acid	Jasmonic acid ~ treatment + leaf pool	5	-121.814	39.138	**<0.001**	2.672	23	**0.014**	8.338	6	**<0.001**	-	-	-
Salicylic acid	Salicylic acid ~ treatment	4	198.287	19.047	**<0.001**	5.031	23	**<0.001**	-	-	-	-	-	-

4 DISCUSSION

The chemistry of the host plant can shape plant-herbivore interactions at many levels up to the herbivore population (Barbour et al. 2015; Richards et al. 2015). Black poplar trees possess a set of direct and indirect chemical defenses, consisting of partly constitutive and partly inducible compounds. While some of these compounds are known for their anti-herbivore-function, much less information is available about the specificity of their induction by herbivores, especially when analyzing several members of a single feeding guild. However, studies investigating specificity of induced resistance and specificity of effect are of great importance to understand how herbivore communities on trees are assembled. This doctoral thesis aimed to investigate the interaction of black poplar with the poplar-associated lepidopteran species *Amata mogadorensis*, *Lymantria dispar* and *Laothoe populi* as well as two leaf beetle species *Phratora vulgatissima* and *Chrysomela populi*. The focus was on the specificity of black poplar defenses to the different herbivore species as well as its consequences.

4.1 INDUCIBILITY OF BLACK POPLAR DEFENSE METABOLITES

Herbivory is well known to alter the metabolome of plants. Poplar trees are no exception (reviewed e.g. in Philippe and Bohlmann 2007; Ralph 2009). Many of the metabolomic changes involved are controlled by phytohormones, which also change in their levels upon herbivore detection (Erb et al. 2012). In the course of this thesis black poplar was found to respond to herbivore feeding with an induction of JA in the attacked leaf **(manuscripts I, III, IV)** as well as in surrounding tissues. In contrast, SA concentrations remained unaltered **(manuscripts I, III, IV)**. The lack of response of SA to herbivory was also previously observed in black poplar (Boeckler et al. 2013; Lackner et al. 2019; Clavijo McCormick et al. 2019) although SA induction after herbivory are known as well (Clavijo McCormick et al. 2014b). This shows that a strict classification of SA as an anti-herbivore signal is difficult. The uncertainties might arise because plant-herbivore-interactions often involve microorganisms as a third player (Eberl et al. 2018) or at least as herbivore-associated microbial communities (Acevedo et al. 2015). Some herbivores exploit microbes for their advantage, thus influencing the plant defense response (Chung et al. 2013). The investigation of the role of microbial communities in plant-herbivore interactions is just emerging and should reveal more in-depth knowledge (Acevedo et al. 2015; Harun-Or-Rashid and Chung 2017). Microbes might be important players in the response of plants to insect egg deposition, which shows high similarities to pathogen-related responses (Hilker and Fatouros 2015), although insect eggs can also

cause a hypersensitive response without microbe involvement (Voirol et al. 2020). However, in both cases the SA pathway is triggered which explains the observed elevated SA levels after *C. populi* egg deposition **(manuscript IV)**. The activation of a hypersensitive response after egg deposition might prevent the growth of pathogens at the oviposition site. Whether this response is controlled by poplar or a result of manipulation by *C. populi* eggs is unclear yet.

The activity of black poplar Kunitz-type trypsin protease inhibitors generally increased subsequent to herbivory **(manuscript I, III, IV),** correlated with significant increases in the transcript abundance of several KTI genes **(manuscript II)**. The positive response of protease inhibitors in poplar to mechanical damage and herbivory are well described (Bradshaw et al. 1990; Haruta et al. 2001; Muller et al. 2019), although not always consistent (Rubert-Nason et al. 2015). KTI activity following herbivory was also reported in black poplar (Ma et al. 2011). KTIs are jasmonate dependent (Haruta et al. 2001; Muller et al. 2019) and therefore an induction of KTIs after herbivory in black poplar was expected. However, an increase in KTI activity in adjacent systemic leaves was not observed **(manuscript I),** which was counter to most other poplar studies (Ma et al. 2011; Major and Constabel 2006, but see Rubert-Nason et al. 2015). This shows that KTI activity is influenced by more than just herbivore damage. The genotype and nutrient status also have influence (Rubert-Nason et al. 2015), although these factors were not directly addressed within this thesis. However, KTIs present an effective anti-herbivore defense in black poplar, since they increase the time of herbivore development and consequently herbivore exposure to natural enemies (especially in combination with HIPVs). Therefore, this type of biochemical defense is probably most effective against larval stages of herbivores.

This thesis investigated herbivory-triggered changes in black poplar VOC emission **(manuscript I)** as well as potential priming effects by HIPVs **(manuscript III)**. Herbivory by the species tested in **manuscript I** changed the emission of volatiles quantitatively by increasing compounds belonging to the groups of terpenes and nitrogenous volatiles. Similar observations were made after *L. dispar* herbivory in **manuscript III**. Terpenes and nitrogenous volatiles were described to be regulated by jasmonates (Boland et al. 1995; Luck et al. 2016; Semiz et al. 2012), and therefore increased concentrations after herbivory seem reasonable. However, while these two volatile groups showed similar induction patterns in **manuscript I** and **manuscript III**, the patterns of aromatic- and green leaf volatiles were less similar. While the emission of aromatic volatiles increased significantly after herbivory for all tested herbivore species in **manuscript I,** there was no significant increase after *L. dispar* herbivory in **manuscript III,** only

a trend. In contrast, while *L. dispar* herbivory increased GLVs during the priming experiment **(manuscript III)**, neither the lepidopteran species *L. dispar* and *L. populi* nor the leaf beetles *P. vulgatissima* and *C. populi* caused significant changes during the specificity experiment **(manuscript I)**. Since herbivore-triggered inductions of GLVs and aromatic compounds were previously observed for *L. dispar* and *P. vulgatissima* in black poplar (Clavijo McCormick et al. 2014b; Unsicker et al. 2015), the inconsistencies between the results of the two experiments might arise from the number of VOCs in each group, which comprised fewer compounds in **manuscript III**. However, although herbivory changed major parts of black poplar's VOC bouquet, no significant changes were observed in the non-volatile compounds of leaves exposed to headspace volatiles of *L. dispar*. Certain as yet unknown compounds within the herbivore-triggered volatile bouquet caused changes in the concentrations of salicin and catechin after a subsequent second herbivory event **(manuscript III)**. Future studies could try to clarify which individual black poplar volatiles or mixtures of them contribute to priming.

Contrary to the other groups of herbivore-related chemicals investigated in black poplar, the inducibility of salicinoids by herbivores could not be clearly demonstrated. Inductions were mainly observed for the simplest salicinoid salicin, while the concentrations of the more complex salicinoids, salicortin and homaloside D, were mostly not significantly increased **(manuscript I, III, IV)**. It must to be noted though that herbivory by all tested herbivore species led to small non-significant increases in salicortin and homaloside D concentrations in adjacent non-damaged leaves **(manuscript I)**. Slight increases in salicortin concentrations were also observed in black poplar leaves exposed to the volatile blend of herbivore-infested leaves **(manuscript III)** and were shown in other black poplar studies as well (Boeckler et al. 2013; Lackner et al. 2019). Such small increases might nonetheless influence the performance of herbivores on black poplar as shown for *L. dispar* in **manuscript III**. The inconsistent results of salicinoid inducibility have long been discussed in the literature and it is believed that factors like genotype, ontogeny and nutritional status are far more influential on salicinoid concentrations than herbivory (reviewed in Boeckler et al. 2011). From the salicinoid data in this thesis, two major points can be extracted. First, when discussing induction of salicinoids in poplar, interpretations regarding the simple salicinoid salicin must be separated from complex salicinoids like salicortin and homaloside D. This is because the induction patterns are different with salicin usually showing a stronger response to herbivory **(manuscript I, III)**. Second, the complex salicinoids show no significant short-term increases in their concentration as shown in **manuscript I**, which is congruent to the results of other black poplar studies (Boeckler et al. 2013; Lackner et al. 2019). The differences in salicinoid inducibility may hint

toward differential ecological roles of these compounds. The results of a study performed by Ruuhola et al. (2001) indicated the 6-hydroxy-2-cyclohexen-on-oyl (HCH) moiety to be the key component in the anti-herbivore activity of salicinoids, but only the complex salicinoids possess this structure. It is speculated that salicin might just be an intermediate in their synthesis (Babst et al. 2010). A more recent study highlighted differences in the biosynthetic pathways of salicin and more complex salicinoids (Babst et al. 2010; Fellenberg et al. 2020). The hypothesis of a different ecological role is strengthened by the observation of a strong tendency towards increased salicin levels in leaves exposed to *C. populi* eggs, but neither salicortin nor homaloside D responded in a similar way **(manuscript IV)**.

4.2 SPECIFICITY OF HERBIVORE-INDUCED RESPONSES IN BLACK POPLAR

4.2.1 Specificity of herbivory-triggered defense response

Since anti-herbivore defenses in plants are known to incur costs (Onkokesung et al. 2016), the development of defenses tailored to the attacking herbivore species is reasonable. In the literature, evidence for herbivore-species-specific defenses in plants was established for herbs (Agrawal 2000; Chung and Felton 2011) as well as for trees (Fields and Orians 2006; Hartley and Lawton 1987; Xiao et al. 2019). Black poplar also showed herbivore-dependent defenses **(manuscript I, II)**, and furthermore differential responses to herbivory and oviposition **(manuscript IV)**, which will be discussed separately (see chapter 4.2.2). The specificity of defense responses was also dependent on the signaling type (local versus systemic induction). For example, in **manuscript I** JA responded to herbivory in damaged local leaves in a non-species specific manner. Response specificity, however, was evident in the non-damaged systemic leaves, where leaf beetles caused stronger JA induction than caterpillars. In general, a true herbivore-specific response (a different response to each herbivore species) was not observed in black poplar defenses. Rather differences were found at the level of herbivore order (Lepidoptera versus Coleoptera). Similar observations have been made before, but these studies compared only one species from each order (Chung and Felton 2011; Moreira et al. 2015; Nguyen et al. 2018; Unsicker et al. 2015). The use of multiple herbivore species revealed the pattern of differential plant responses to lepidopterans and coleopterans more clearly, especially for the induction of JA, the activity and transcript abundance of KTIs, and the release of some groups of volatiles. The pattern of stronger KTI activity after herbivory by the leaf beetle *P. vulgatissima* was observed in local damaged leaves **(manuscript I)**. The transcription of most relevant KTI genes measured within **manuscript II** showed clear and significant differences between *P. vulgatissima* and the two lepidopteran herbivores and

therefore supported the activity patterns. In a similar way, black poplar orchestrated herbivore-specific defense responses in the emission of VOCs, especially for compounds belonging to terpenes and nitrogenous volatiles. Similar to the induction patterns of JA and KTIs, the differences in terpene emission after herbivory were herbivore order-specific rather than species-specific. The pattern of herbivory-triggered changes in the emission of nitrogenous volatiles was slightly different from changes in terpene emission, but again a leaf beetle, *C. populi,* caused the strongest increase in their emission. Herbivore-specific volatile emissions are repeatedly described in the literature (Danner et al. 2018; Hare and Sun 2011; Moreira et al. 2013; Pinto-Zevallos et al. 2018). Also differences between lepidopterans and beetles were reported before (Hare and Sun 2011), even in black poplar trees, where a leaf beetle also used in **manuscript I**, *P. vulgatissima,* was observed to be more volatile-inducing than the lepidopteran *L. dispar* (Clavijo McCormick et al. 2014b; Unsicker et al. 2015). However, the described differences derived from two experiments and are therefore difficult to compare. In **manuscript I,** higher volatile emission was observed again for two leaf beetle species and within a single experiment, which strengthens the validity of the results discussed in (Unsicker et al. 2015). In contrast to KTI expression and the emission of terpenes and nitrogenous volatiles, the herbivore identity had no significant influence on the induction of salicin, aromatic volatiles or GLVs. These results present an excellent example of the complexity behind the specificity of anti-herbivore defense responses, with one compound group (and even individual compounds within a group) showing different patterns than others. The question about herbivore-specific defense responses in plants must therefore be addressed with care.

Observations of herbivore-specific defense responses raise questions about the mechanism behind their specificity. KTIs are thought to be induced by herbivore damage whereby wounding seems to be the important part and the presence of herbivore regurgitants or oral secretions has less strong effects (Major and Constabel 2006; Philippe et al. 2009). However, there was no correlation between KTI activity or transcript abundance and herbivore damage in black poplar (data not published). Therefore other factors like herbivore feeding pattern might be influential as already suggested by Green and Ryan (1972). Indeed, the feeding pattern of *P. vulgatissima* is very unique, since it arises from many irregular lesions, which occur mainly on the abaxial surface of the leaf blade (although feeding on the adaxial surface and leaf perforations was also observed, but less intense). Ultimately the leaf is skeletonized, which is very distinct from the pattern observed from feeding of late stage lepidopteran herbivore species, which cut out large chunks of the leaf blade (**Fig. 1, manuscript I**). Investigations on feeding

pattern are not easy to realize, since the lesions caused by *P. vulgatissima*, for example, are very small and hard to estimate in terms of area or number. A study on black poplar by Clavijo McCormick et al. (2014a) succeeded to combine these two factors for younger instar gypsy moth larvae. They found significant correlations between a factor called "feeding intensity" and certain volatiles emitted by black poplar. Even though it would be much more challenging to analyze the feeding intensity of *P. vulgatissima* in a similar way, this factor is still worth investigating since such a feeding mode could well test the influence of damage parameters on the activity of poplar KTIs. Also, the results about the specificity of herbivore-triggered black poplar VOC emission raise questions about its regulation. Here again it could be argued that the feeding pattern is responsible for the differential induction. However, in **manuscript I** the poplar leaf beetle *C. populi* also caused increased emissions of terpenes and nitrogenous volatiles although feeding pattern and amount of damage caused by this species are more similar to damage patterns inflicted by lepidopteran caterpillars with the removal of large chunks of leaf foliage. Although the role of damage type in determining plant VOC emission pattern was significant as in other studies (Clavijo McCormick et al. 2014a; Gouinguene et al. 2003; Clavijo McCormick et al. 2019; Wang et al. 2019a), its role in causing the differences between beetles and lepidopterans still has to be elucidated. In a similar way, herbivore oral secretions may explain herbivore order-specific volatile emissions (Schmelz et al. 2009; Sobhy et al. 2017) based on a component that is order-specific. It is known that lepidopteran larvae possess elicitors located in oral secretions consisting of regurgitant from the anterior part of their gut and secretions from labial and mandibular glands, which are the most copious secretion (Acevedo et al. 2015; Felton et al. 2014b). Additionally, phytohormones located in their saliva glands can modulate plant responses (Acevedo et al. 2019). In contrast, leaf beetles do not possess labial glands (Chung et al. 2013). It was described earlier that larvae and adults of chrysomelid beetles possess maxillary glands instead. Amylases were not found within the maxillary glands of dissected leaf beetle species, suggesting that they may not function as salivary glands (Srivastava 2009). The absence of salivary glands might force chrysomelid beetles to use more regurgitant during feeding, offering one possible explanation for the patterns we observed. Analyses of the chemical content of regurgitants are increasing with the first beetle regurgitome being published recently (Gedling et al. 2018). It is very likely that differences in the microbiome of the insect are involved in the differences in the defense response of black poplar. It is well-known that microbes present in secretions of insect glands or gut of insects can modulate plant defense responses (Acevedo et al. 2017; Chung et al. 2013; Mason et al. 2019). Microbial communities and their involvement in plant-herbivore interactions is a comparably new and interesting branch of research and will shed more light onto herbivore-specific

defense responses in plants.

4.2.2 Herbivory-triggered response versus egg-triggered response

The experimental data of this thesis demonstrated significant differences between herbivory- and egg-induced phytohormonal changes in black poplar leaves **(manuscript IV)**. While herbivory caused a strong burst in JA, it did not affect SA, which on the other hand increased beneath the area where egg material was experimentally applied. JA was less responsive to insect eggs and the concentrations in leaves exposed to egg material were very low compared to the concentrations in leaves experiencing herbivory **(manuscript I, manuscript IV)**. Therefore, at least for *C. populi* the data suggest that black poplar responds to herbivory by activating the JA pathway while herbivore eggs mainly activate the SA pathway although more studies with additional herbivore species are needed to reinforce this pattern before general conclusions can be drawn. It is already known that plant responses to herbivory as well as responses to herbivore eggs can be herbivore-specific (Agrawal 2000; Chung and Felton 2011; Mumm and Hilker 2006; Mumm et al. 2005). The data of this thesis show that different developmental stages of the same herbivore species can cause distinct poplar defense responses. The dependence of black poplar defense responses on the herbivore developmental stage is known for another herbivore species, *L. dispar,* though different larval stages were investigated and not eggs (Clavijo McCormick et al. 2014a). Similar observations were reported in other plant-herbivore systems (Takabayashi et al. 1995; Yoneya et al. 2009). It was suggested that the amount and type of damage, which differs between herbivore stages, is involved into the observed patterns. However, this cannot explain the data of **manuscript IV**; since eggs alone do not damage the plant in a way similar to herbivory (*C. populi* glues its eggs on the leaf without causing observable lesions). On the other hand, the absence of tissue wounding during oviposition might be a potential reason for the differential response. Evidence from the literature suggests that oviposition without wounding mainly triggers the SA pathway (Bertea et al. 2020; Reymond 2013). As another possibility, the data obtained suggest either different elicitors occurring in the saliva/regurgitant and ovipositional secretions or varying concentrations of the same elicitor in different herbivore secretions. Elicitors are thought to be the main reason for the specificity of plant defense response to herbivory and oviposition. Many elicitors are found in the oral secretions of insects, although information about elicitors involved into the oviposition process is still scarce (reviewed in Furstenberg-Hagg et al. 2013; Hilker and Fatouros 2015; Hilker and Meiners 2010). Also differences in herbivore microbial communities may play a role (Acevedo et al. 2017; Acevedo et al. 2015; Bertea et al. 2020, but see Voirol et al. 2020). For *C. populi,* it is not clear yet which elicitors are responsible for

oviposition-induced plant responses, where they occur and to which extent microbial communities accompanying ovipositional secretions play a role. Also, there is a lack of knowledge of the poplar receptors involved in detecting the presence of herbivores as well as their eggs. Beside efforts to find specific elicitors and receptor proteins, future studies have to test the observed patterns with other herbivore species to disentangle the effects of herbivory and oviposition. However, despite significant differences in phytohormone responses there were also similarities in plant responses to herbivory and oviposition, such as in the induction patterns of salicinoids. In both cases, salicin concentrations slightly increased after the treatment while the complex salicinoids remained mostly unaffected **(manuscript I, manuscript IV)**. This observation together with the observation of differential phytohormone patterns after herbivory compared to oviposition again raises questions about the mechanisms behind salicinoid induction. Although the involvement of phytohormones in the regulation of plant defense is indisputable, transcriptional analyses in *Arabidopsis* showed that in some cases such responses can also be elicited independent of phytohormonal regulation (Little et al. 2006). It is possible that the regulation of salicinoids presents a similar case. Unfortunately, direct studies showing the effect of phytohormones on poplar salicinoid concentrations are still missing and could be the focus of future research.

4.3 CONSEQUENCES OF POPLAR DEFENSE FOR INSECT HERBIVORES

The complexity of anti-herbivore chemical defenses in plants impacts herbivores on several levels. Constitutive chemical diversity affects herbivore diversity, herbivore specialization and natural enemies of herbivores (Richards et al. 2015), while the diversity of plant defense responses can also shape herbivore community structure (Barbour et al. 2015; Whitehead et al. 2021), with different effects within and between host plants. In the experiments performed within the framework of this thesis, the defense response of black poplar was found to be partly non-herbivore-specific and partly herbivore-specific. Both types of specificity can influence poplar-associated herbivore species and species at higher trophic levels in their own way.

The most prominent defense compounds in black poplar that did not show a herbivore-specific response were the salicinoids. Nonetheless, salicinoids were recently suggested to have a high capacity for evolution, because of their high genotypic variation (Barker et al. 2019). Thus, insect community patterns in trees may already be shaped through variable constitutive tree defenses without specific induced defense responses. Depending on their specialization level, herbivores deal with these defense

compounds in different ways, as shown in the two performance experiments reported within this thesis. In **manuscript III**, we found a negative correlation between the concentration of salicortin and the performance of the generalist *L. dispar*. Variations in salicortin concentrations lead to strong variations in *L. dispar* larval performance. Consequently, the sensitivity of *L. dispar* to salicinoids affects the distribution of this herbivore species, leading to avoidance behavior. During the experiment described in **manuscript I**, the larvae usually avoided younger leaves even within their designated leaf pools (unpublished observation). **Manuscript III** suggests that the changes in salicin due to priming caused the avoidance of HIPV-exposed leaves. This avoidance of salicinoids indicates how susceptible a herbivore species is to this major class of defenses. By feeding on mature leaves, contact with salicinoids is reduced because of the ontogenetic variation in concentration, but this benefit comes with the disadvantage of reduced nutritional value (Barbehenn et al. 2015). The avoidance of salicinoids by generalist herbivores like *L. dispar* creates niches that could be inhabited by specialist herbivores possessing the ability to cope with these defensive chemicals. An example of such a specialist is *C. populi*, a species that feeds especially on juvenile leaves of black poplar, whose larvae seem not to suffer any disadvantages in performance from this choice (**manuscript IV**), perhaps because of their ability to sequester salicinoids for their own protection against predators (Pasteels et al. 1983). However, the observation that this species avoids oviposition on young leaves shows the complexity of plant-herbivore-interactions. Other factors like the avoidance of con-, hetero- and allospecific competition, leaf loss by competitors, predation by enemies as well as the provision of shelter might be involved in the choice of older leaves for egg deposition. Such clear herbivore preferences indicate that different developmental stages probably have different requirements for survival and performance.

Another observation we made on black poplar salicinoids with possible impact on herbivore communities was the lack of inducibility of this compound class. Inducible plant defenses can mediate intra- and interspecific competition between herbivore species when one species indirectly deters or suppresses the development of a second herbivore species arriving later. This argument is especially strong when tolerant specialist and susceptible generalist herbivores are involved. However, the constitutive character of most salicinoid defenses in black poplar does not facilitate such interactions (data not published) even though salicin itself was indeed inducible. However, it was recently shown that other compounds like flavones and especially synergistic mixtures of phenolic compounds have much more impact on *L. dispar* (Wang et al. 2019b). Our knowledge about synergies of plant defense metabolites is still scarce, but a meta-analysis recently suggested that mixtures of plant defenses might

be more effective than single compounds (Richards et al. 2016). Investigations on the potential synergies of defense metabolites in black poplar would be very welcome.

The strongest patterns of herbivore-specific defense responses in black poplar were observed for KTIs and VOCs. While the herbivore-specific induction of KTIs could shape herbivore communities via plant-mediated indirect interactions between herbivores, the specific induction of certain VOCs would affect priming and the attraction of natural enemies. In poplar, direct tests addressing the relationship between KTIs and herbivore performance are scarce, but data of biochemical studies suggests that these protease inhibitors have an influence (Major and Constabel 2008; Philippe et al. 2009). KTIs have extremely diverse protease targets and hence collectively have negative effects on a broad range of phytophagous pests and pathogens (Major and Constabel 2008). However, it is reasonable to expect that some herbivore species are more susceptible than others. Even if inducibility were not herbivore-specific, differences in KTI susceptibility could already influence herbivore distribution within and (dependent on their mobility) between trees. Susceptible herbivore species arriving at a previous feeding site would suffer disadvantages due to induced KTI activity. The stronger KTI activity observed after leaf beetle attack could multiply this impact. Unfortunately, the direct effect of KTIs on herbivore performance was not measured in **manuscript IV**. Future experiments using KTI enriched diets could help test the significance of these defenses. Mixtures of KTIs with other poplar defense metabolites should also be considered to test for synergistic effects.

The herbivore-specific induction of VOCs, such as terpenoids and nitrogenous volatiles in black poplar, as described in **manuscript I**, might shape herbivore communities on a much wider scale than KTIs through volatile-mediated priming of anti-herbivore defenses. The greater emission of volatiles caused by leaf beetle feeding would lead to stronger primed responses within- and between host plants. Priming potential has been reported for a group of non-specifically induced VOCs, the GLVs (Frost et al. 2008), and for a group of specifically-induced VOCs, terpenes (Arimura et al. 2000). Although not experimentally tested within this thesis, the volatile profile of egg-infested leaves might lead to priming as well. Herbivores arriving on primed plants might be repelled or suffer other disadvantages as shown for *L. dispar* in **manuscript III**. In this way, stronger VOC emission after leaf beetle feeding might have a negative effect on subsequently arriving herbivores in a greater area around the feeding spots. Future tests should therefore aim to investigate the influence of species identity, developmental stage and spatial extent on priming events in poplar. Additionally, herbivore-specific VOC emission might affect

natural enemies involved in indirect defense against herbivores. Some of the most important VOCs in poplar are nitrogenous volatiles, which were induced in a herbivore-specific manner. Since natural enemies like parasitic wasps partly orient to such compounds (Clavijo McCormick et al. 2014b), the stronger emission of nitrogenous volatiles, as observed after *C. populi* feeding can increase the attraction of natural enemies. However, enemies such as predators might target competing herbivore species, especially when the inducing species sequesters plant metabolites as defenses, as described for *C. populi* on poplar. In this way, volatile emission might even benefit the inducing herbivore. Additionally, certain HIPVs can directly repel herbivores, preventing them from long-term inhabitation on infested areas (Dicke and van Loon 2000). Thus, the net effects of plant volatile emission are very complex and there are many open questions. Some address the composition of odor blends necessary for the recognition by natural enemies, while others address how the co-occurrence of several herbivore species will impact the HIPV blend released (reviewed by (Clavijo McCormick et al. 2012).

4.4 CONCLUSION AND OUTLOOK

This thesis addressed the specificity of chemical anti-herbivore defenses in black poplar and speculated about the consequences for herbivores. The results fill gaps in previous studies caused by using insufficient numbers of herbivore species without controlling for feeding guild and herbivore specialization level. We demonstrated that black poplar exhibits specificity in some but not all chemical defense responses. The herbivore-specific defenses include protease inhibitors and some groups of VOCs. Intriguingly, the response differed most often at the level of herbivore order (Coleoptera versus Lepidoptera) rather than the level of the herbivore species. Such specificity might be involved in mediating herbivore-herbivore interactions over short and (in the case of VOCs) long distances. To shed more light on the aspect of plant-mediated herbivore-herbivore-interactions future research must aim to answer a basic question about whether plants or herbivores are “in charge” of the situation. Are the herbivores manipulating plant defenses or just passively responding to them? If herbivores can manipulate plants, which mechanisms are involved? In contrast to induced defenses, this thesis found that a large proportion of chemical defenses were constitutive with nonetheless striking effects especially on generalist herbivores like *L. dispar*. Specialist herbivores like *C. populi* seem to be less affected but nonetheless avoid the juvenile tissues with the highest concentrations when ovipositing for reasons not fully understood yet. However, their ability to sequester salicinoids might allow *C. populi* to feed on nutrient-rich juvenile poplar tissues avoided by generalists like *L. dispar*.

At this point it must be noted that the results obtained in this thesis originate from experiments conducted in a controlled environment and therefore may not accurately replicate the interactions occurring in natural systems. Controlled environments typically lack diverse microbial communities and fungi, do not accurately reproduce factors like light, nutrient and water supply and seasonal change, and do not provide the massive number of other plants or animals constantly interacting with the poplar-herbivore system. Despite this, studies in controlled environments are needed to identify important patterns. The isolation from certain less controllable environmental conditions and the possibility to manipulate them often renders patterns visible in the first place. However, the results obtained by experiments in controlled environments should be later be verified in the field if we want to understand how the systems we investigate really function.

5 SUMMARY

The interactions of plants and their herbivores are complex in part because of the specificity of plant defense responses. The specificity of anti-herbivore defense helps plants reduce their physiological and ecological costs while at the same increasing the effectiveness of defenses. Although the specificity of anti-herbivore defenses has been investigated before, most of the studies in the literature tested only two herbivore species often mixing feeding guilds and herbivore specialization level, making it difficult to detect patterns. This thesis investigated the specificity of phytochemical anti-herbivore defenses in a boreal tree species, the black poplar (*Populus nigra* L.), using up to five herbivore species belonging to the same feeding guild, but with different levels of specialization. It mainly focused on defense specificity based on herbivore identity, but also investigated induction profiles in response to different herbivore developmental stages (adult stage versus egg stage). In addition, the effects of black poplar defense on two herbivore species, the generalist lepidopteran *Lymantria dispar* and the specialist coleopteran *Chrysomela populi* (L.), were analyzed with performance experiments. All in all, the main defense of black poplar, the salicinoids, showed no herbivore-specific induction and little induction in general. In contrast, Kunitz-type trypsin protease inhibitors (KTIs) as well as two groups of volatile organic compounds responded in an herbivore-specific fashion. Interestingly, the greatest differences in black poplar defense specificity were observed at a higher taxonomic level. Coleopteran attack resulted in higher transcript abundance and activity of KTIs and higher emission of terpenes and nitrogenous volatiles, compared to lepidopteran attack. Herbivory and oviposition on black poplar leaf foliage by *C. populi* resulted in different phytohormone responses with jasmonic acid induced in response to herbivory and salicylic acid induced in response to oviposition. However, no other measured metabolites were affected by *C. populi* eggs, although the salicinoid salicin showed a strong tendency towards increased concentration. Salicin was also the only metabolite significantly affected by priming. The salicinoid salicortin was shown to negatively affect *L. dispar* performance, which consequently avoided leaves with increased concentrations. It has been known for some time that poplar juvenile leaves contain higher salicinoid concentrations than mature leaves. The specialist *C. populi* nonetheless preferred feeding on juvenile poplar leaves, but adult females avoided those leaves for oviposition. This decision was, however, not reflected in any improvement in larval performance under the experimental conditions. The data obtained highlight the complexity of plant-herbivore-interactions and show that both, herbivore-specific defenses as well as non-specific defenses have the potential to affect the composition of poplar-associated herbivore communities. Herbivore-specific defenses multiply the

effects of poplar defenses and present a mechanism for plants to mediate herbivore-herbivore interactions with generalist herbivores especially at a disadvantage. However, more research on herbivore-specific defenses, particularly on different species and in different environments needs to be conducted to improve our understanding of this topic.

6 ZUSAMMENFASSUNG

Interaktionen zwischen Pflanzen und ihren Herbivoren sind vielfältig und komplex, zum Teil aufgrund der Spezifität der Verteidigung von Pflanzen gegen Herbivoren. Eine Spezialisierung der Verteidigung reduziert ökologische und physiologische Kosten und erhöht gleichzeitig die Effektivität. Obwohl Studien über die Spezifität der Verteidigung bereits durchgeführt wurden, beschränken sich viele davon auf zwei Herbivorenarten und vermischen häufig Fraßgilden und das Spezialisierungslevel der Herbivoren, was allgemeine Muster schwer erkennbar macht. Diese Dissertation untersuchte die Spezifität der chemischen Verteidigung gegen Herbivoren in einer borealen Baumart, der Schwarzpappel (*Populus nigra* L.). Hierfür wurden bis zu fünf Herbivorenarten aus der gleichen Fraßgilde verwendet, die verschiedene Spezialisierungsgrade besitzen. Die Dissertation konzentrierte sich hauptsächlich auf die Spezifität der Verteidigung in Abhängigkeit der Herbivorenart, unteruchte jedoch auch die Abhängigkeit der Induktionsprofile vom Herbivoren-Entwicklungstadium (adultes Stadium gegen Ei-Stadium). Anhand von Entwicklungsexperimenten wurden Auswirkungen des Verteidigungverhaltens der Schwarzpappel auf zwei Herbivorenarten, die generalistische Mottenart *Lymantria dispar* (L.) und die spezialistische Blattkäferart *Chrysomela populi* (L.) untersucht. Zusammengefasst besaßen die Hauptabwehrstoffe der Schwarzpappel, Salicinoide, keine herbivorspezifischen Induktionen und hatten hauptsächlich einen konstitutiven Charakter. Im Gegensatz dazu zeigten Kunitz-Typ Trypsin Protease Inhibitoren (KTIs) und zwei Gruppen von Pflanzenduftstoffen herbivorspezifische Reaktionen. Interessanterweise wurden die größten Induktionsunterschiede nicht auf der Arten- sondern auf der Ordnungsebene festgestellt. Im Vergleich zu Mottenfraß resultierte Blattkäferfraß in höherer Transkriptabundanz und Aktivität von KTIs und führte zu einem erhöhten Ausstoß von Terpenen und stickstoffhaltigen Duftstoffen. Fraß und Eiablage von *C. populi* auf Schwarzpappelblättern erzeugte verschiedene phytohormonelle Antworten. Der Fraß induzierte das Hormon Jasmonsäure (JA) während die Eiablage zu einer vermehrten Ausschüttung von Salicylsäure führte. Jedoch erzeugte die Eiablage keine signifikanten Unterschiede in den weiterhin gemessenen Metaboliten. Die einzige Ausnahme bildete das Salicinoid Salicin, das direkt unterhalb der Eipakete erhöhte Konzentrationen besaß. Salicin war auch das einzige Metabolit, das signifikant durch Priming beeinflusst werden konnte. Ein weiteres Salicinoid, Salicortin, wirkte sich nachweislich negativ auf die Entwicklung von *L. dispar* Raupen aus. Diese vermieden Blätter mit erhöhten Konzentrationen an Salicinoiden, wie sie vor allem in juvenile Blattgeweben zu finden sind. *C. populi* bevorzugte es, an juvenilen Blättern zu fressen. Jedoch vermieden adulte Weibchen die juvenilen Blätter bei der Eiablage obwohl diese Entscheidung keine Auswirkungen auf die

Larvenentwicklung hatte. Die innerhalb dieser Dissertation gesammelten Daten unterstreichen die Komplexität von Pflanzen-Herbivoren-Interaktionen und zeigen, dass sowohl unspezifische- als auch artenspezifische Verteidigungen der Schwarzpappel das Potenzial besitzen, assoziierte Herbivorengemeinschaften zu beeinflussen. Eine herbivorspezifische Verteidigung verstärkt hierbei die Auswirkungen der Verteidigung auf Herbivoren und bietet damit eine solide Basis für indirekte, pflanzenvermittelte Interaktionen zwischen Herbivorenarten. Die Erkenntnisse dieser Dissertation zeigen auch, dass der Verteidigungstyp der Schwarzpappel generalistische und spezialistische Herbivorenarten in verschiedener Weise beeinflusst. Generalisten könnten hierbei größere Nachteile erfahren, als Spezialisten. Jedoch ist unser Verständnis über die Effekte einer herbivorspezifischen Verteidigung auf Herbivorengemeinschaften noch immer unscharf und benötigt weitere Studien.

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9 EIGENSTÄNDIGKEITSERKLÄRUNG

Hiermit erkläre ich, dass mir die geltende Promotionsordnung der Biologisch-Pharmazeutischen Fakultät der Friedrich-Schiller-Universität Jena bekannt ist. Entsprechend § 5 Abs. 4 der Promotionsordnung bestätige ich, dass ich diese Dissertation selbst angefertigt habe und keine Textabschnitte eines Dritten oder eigener Prüfungsarbeiten ohne Kennzeichnung übernommen habe. Weiterhin habe ich alle benutzten Hilfsmittel und Quellen angegeben. Personen, die mich bei der Erhebung und Auswahl des Materials sowie bei der Erstellung der Manuskripte unterstützt haben, sind in der Auflistung der Manuskripte (Kapitel 2, Overview of Manuscripts) genannt oder werden, im Falle von Beiträgen geringeren Ausmaßes, in der Danksagung genannt. Ich habe keine Hilfe eines Promotionsberaters in Anspruch genommen und es wurden im Zusammenhang mit dem Inhalt der Dissertation keine Geldwerte oder Leistungen unmittelbar oder mittelbar an Dritte weitergegeben. Die Dissertation wurde nicht bereits zuvor als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung eingereicht. Weiterhin wurde keine gleiche, in wesentlichen Teilen ähnliche oder andere Abhandlung als Dissertation bei einer anderen Hochschule eingereicht.

Jena, den 20.08.2021

Thomas Fabisch

10 SUPPLEMENTARY DATA

10.1 MANUSCRIPT I – SUPPLEMENTARY DATA

SPECIFICITY OF HERBIVORE DEFENSE RESPONSES IN A WOODY PLANT, BLACK POPLAR (*POPULUS NIGRA*)

Supplemental data

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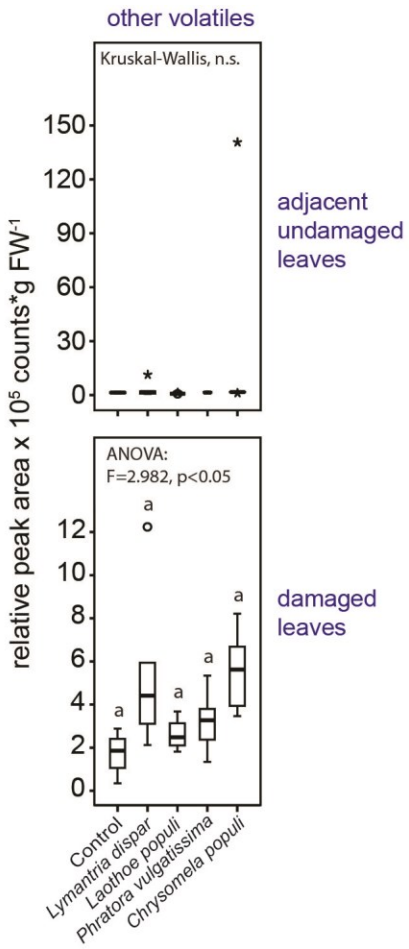

Fig S1 Effect of damage by four herbivore species on the relative amounts of the volatile group "others" emitted from damaged and adjacent undamaged leaves of young *Populus nigra* trees as compared to equivalent leaves from non-infested control trees.

Samples were collected 44 h after infestation with caterpillars of two lepidopteran species, *Lymantria dispar* and *Laothoe populi*, adults of two coleopteran species, *Phratora vulgatissima* and *Chrysomela populi*, and from untreated control plants. The box plots represent median ± 1.5 x interquartile range for n=5 tree replicates. Letters indicate the results of *Tukey-Kramer* (ANOVA) and *Dunn's post hoc testing* (Kruskal-Wallis). Circles indicate outliers and asterisks indicate extreme outliers

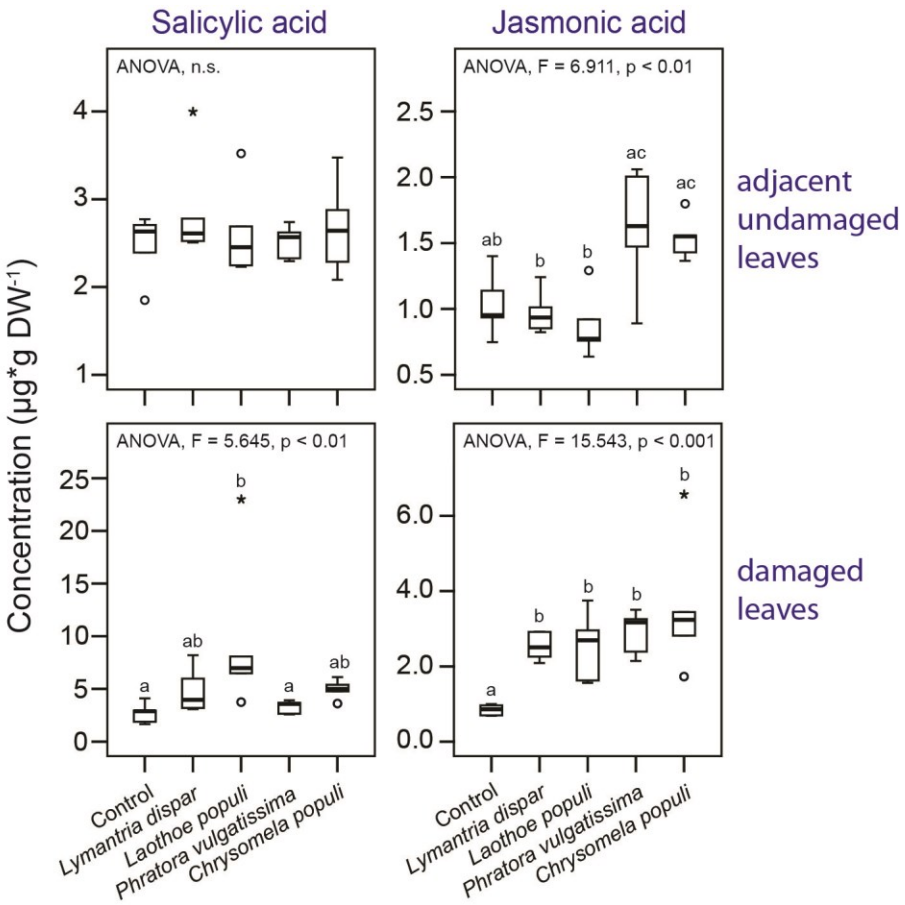

Fig S2 Effect of damage by four herbivore species on the concentrations of the two defense-related phytohormones, jasmonic acid and salicylic acid, in damaged and adjacent undamaged leaves of young *Populus nigra* trees as compared to equivalent leaves from non-infested control trees.

Samples were collected 44 h after infestation with caterpillars of two lepidopteran species, *Lymantria dispar* and *Laothoe populi*, adults of two coleopteran species, *Phratora vulgatissima* and *Chrysomela populi*, and untreated control plants. The box plots represent median ± 1.5 x interquartile range for n=5 tree replicates. Letters indicate the results of *Tukey-Kramer* (ANOVA) and *Dunn's post hoc testing* (Kruskal-Wallis). Circles indicate outliers and asterisks indicate extreme outliers.

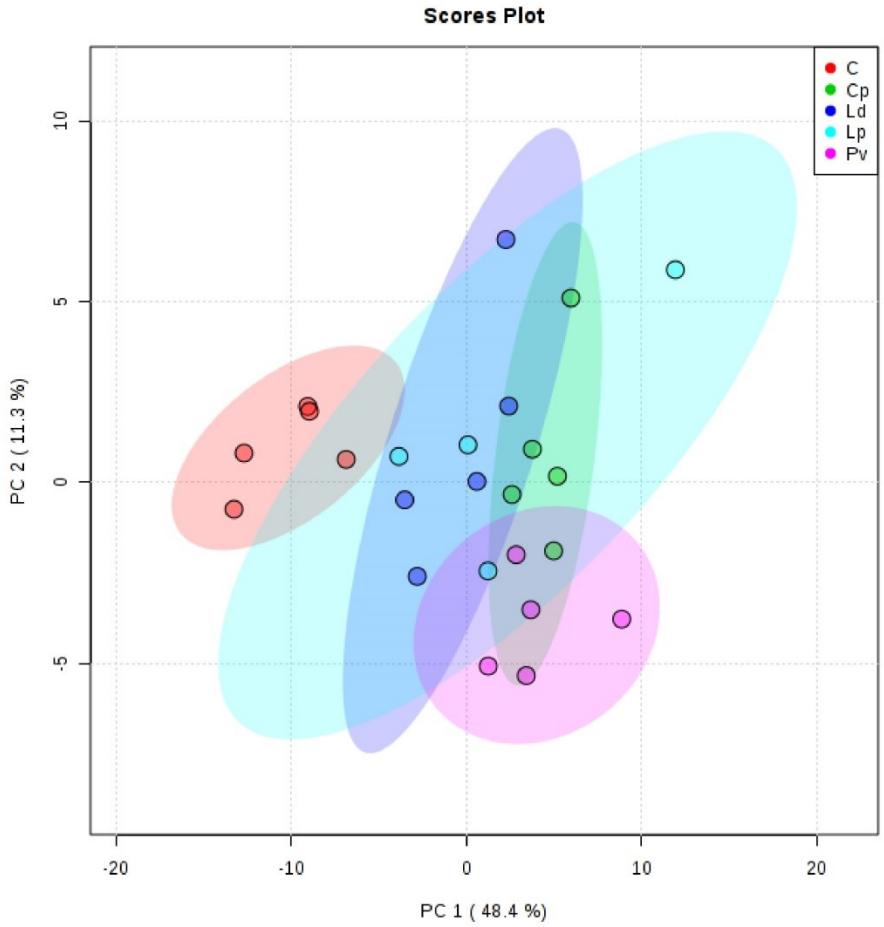

Fig S3: Principal component analysis (PCA) of volatile organic compounds (VOCs) in the headspace of damaged black poplar leaves after herbivory by two lepidopteran species, *Lymantria dispar* (Ld = dark blue curcles) and *Laothoe populi* (Lp = light blue circles), or two coleopteran herbivores *Phratora vulgatissima* (Pv = pink circles) and *Chrysomela populi* (Cp = green circles) compared to the headspace of non-damaged control trees (C = red circles).

Before executing the PCA all variables were transformed (using generalized logarithm transformation) and scaled (mean-centered and divided by the standard deviation of each variable). The score plot shows the first two components with the percentage of variance explained enclosed in parentheses. The plots were produced using the metabolomics platform MetaboAnalyst (https://www.metaboanalyst.ca)

	Control (no herbivore)		*Lymantria dispar*		*Laothoe populi*		*Phratora vulgatissima*		*Chrysomela populi*	
	control for damaged leaves	control for adj. undamaged leaves	damaged leaves	adjacent undamaged leaves	damaged leaves	adjacent undamaged leaves	damaged leaves	adjacent undamaged leaves	damaged leaves	adjacent undamaged leaves
Compound	**mean ± SE**	**mean ± SE**	**mean ± SE**	**mean ± SE**	**mean ± SE**	**mean ± SE**	**mean ± SE**	**mean ± SE**	**mean ± SE**	**mean ± SE**
Monoterpenoids										
α-Pinene*	1114 ± 400	3171 ± 568	1818 ± 277	3802 ± 732	4467 ± 1871	2682 ± 440	4000 ± 838	5461 ± 1552	2560 ± 246	7425 ± 2590
Camphene	354 ± 216	1309 ± 976	789 ± 306	1678 ± 703	307 ± 84	1403 ± 523	1056 ± 885	2273 ± 1309	363 ± 67	1572 ± 1177
1,8-Cineole*	1181 ± 343	5905 ± 1498	11862 ± 2513	5980 ± 1005	56382 ± 40537	6707 ± 1400	32718 ± 14983	12712 ± 3676	24888 ± 2847	16499 ± 5555
β-Ocimene*	217 ± 94	571 ± 167	4398 ± 2389	995 ± 299	7891 ± 4301	1473 ± 983	6259 ± 4063	676 ± 83	7564 ± 2736	1280 ± 636
Linalool*	377 ± 120	349 ± 98	1211 ± 304	465 ± 211	3736 ± 2926	246 ± 80	2827 ± 1122	364 ± 69	2384 ± 336	9972 ± 9067
Linalool oxide 1*	277 ± 84	472 ± 81	2260 ± 824	1175 ± 310	2293 ± 1185	2602 ± 1285	3838 ± 2187	3617 ± 1217	3868 ± 1171	3007 ± 609
Linalool oxide 2	142 ± 46	217 ± 39	613 ± 173	279 ± 59	1385 ± 668	421 ± 95	709 ± 71	679 ± 105	1157 ± 576	559 ± 100
Linalool oxide 3	505 ± 263	1293 ± 335	2065 ± 613	3481 ± 1870	2000 ± 410	2429 ± 563	5003 ± 1487	4662 ± 1643	4209 ± 1222	5137 ± 2014
Camphor*	10034 ± 2859	25462 ± 5010	24642 ± 3516	42482 ± 10202	64247 ± 32289	30372 ± 2976	83187 ± 15863	53856 ± 12000	50873 ± 6616	71991 ± 29825
Borneol*	494 ± 258	1222 ± 314	1920 ± 534	3335 ± 1855	1776 ± 369	2198 ± 545	4941 ± 1539	4420 ± 1592	2512 ± 666	4903 ± 1968
Terpinen-4-ol*	1544 ± 1327	944 ± 294	1236 ± 605	547 ± 240	3117 ± 1034	199 ± 65	1974 ± 1445	743 ± 299	924 ± 126	796 ± 335
α-Terpineol*	157 ± 32	139 ± 32	364 ± 113	182 ± 55	960 ± 203	177 ± 13	708 ± 193	4197 ± 3921	959 ± 190	326 ± 63
β-Citronellol	560 ± 140	823 ± 106	3545 ± 1430	2537 ± 1035	6141 ± 3173	2693 ± 1093	9178 ± 2016	4261 ± 1805	9728 ± 3444	3693 ± 1263
Geranyl acetone	804 ± 169	255 ± 50	1124 ± 284	422 ± 66	18251 ± 17277	757 ± 380	3399 ± 1666	1665 ± 307	2559 ± 967	1183 ± 263
α-Terpinene*	374 ± 156	1201 ± 265	6528 ± 2310	2972 ± 1119	9366 ± 3825	3615 ± 1256	11532 ± 2854	8207 ± 2374	12698 ± 3703	6221 ± 1525
Unidentified monoterpenoid	266 ± 143	107 ± 33	251 ± 76	257 ± 168	823 ± 408	86 ± 14	342 ± 82	159 ± 47	1341 ± 332	166 ± 49
Sesquiterpenoids										
α-Cubebene [(a)]	86 ± 32	141 ± 41	759 ± 177	398 ± 121	1061 ± 299	511 ± 188	3296 ± 1150	1580 ± 269	2057 ± 258	1154 ± 148
(*E*)-β-Caryophyllene [(a)]	158 ± 51	1078 ± 391	1158 ± 409	2050 ± 1108	1233 ± 485	853 ± 562	1251 ± 541	2663 ± 1323	2321 ± 866	3565 ± 1580
β-Copaene [(a)]	105 ± 29	568 ± 144	662 ± 194	487 ± 41	1013 ± 361	1083 ± 614	2741 ± 1268	1844 ± 335	1917 ± 471	1413 ± 322
α-Humulene*	387 ± 179	378 ± 61	2258 ± 827	927 ± 233	33720 ± 32005	2007 ± 874	12489 ± 3593	6775 ± 2431	4527 ± 1018	3718 ± 1013
Naphthalene [(a)]	124 ± 46	883 ± 415	5410 ± 1924	2689 ± 1062	253460 ± 251956	17718 ± 14540	7203 ± 3416	10607 ± 3096	14561 ± 10513	6351 ± 2233
(*E,E*)-α-Farnesene [(a)]	4970 ± 4865	811 ± 339	1656 ± 486	1552 ± 568	1051437 ± 1049852	3193 ± 2083	9279 ± 4075	5034 ± 935	3916 ± 816	4632 ± 1230
Nerolidol*	529 ± 198	614 ± 78	1402 ± 584	445 ± 121	2297 ± 1240	807 ± 258	908 ± 277	410 ± 101	2409 ± 1247	498 ± 86
Guaiol	75 ± 33	322 ± 51	266 ± 70	556 ± 91	761 ± 519	979 ± 300	547 ± 156	1186 ± 627	523 ± 204	1146 ± 401
τ-Cadinol	41 ± 4	180 ± 64	282 ± 93	758 ± 366	585 ± 385	386 ± 148	1092 ± 698	1311 ± 991	237 ± 49	904 ± 477
Unidentified sesquiterpenoid 1	101 ± 31	356 ± 63	2322 ± 694	1224 ± 420	2012 ± 712	1597 ± 726	10375 ± 4345	4823 ± 947	5121 ± 979	3657 ± 807
Unidentified sesquiterpenoid 2	366 ± 82	204 ± 45	1540 ± 489	790 ± 292	1522 ± 612	1148 ± 592	7582 ± 3116	3434 ± 723	3663 ± 893	2600 ± 472
Unidentified sesquiterpenoid 3	178 ± 57	2228 ± 2112	11825 ± 3296	11637 ± 3309	26257 ± 17603	15206 ± 6287	70138 ± 29966	49083 ± 9770	24480 ± 8043	36475 ± 7846
Unidentified sesquiterpenoid 4	1607 ± 678	8604 ± 2234	9313 ± 1183	10600 ± 2361	17828 ± 10205	13245 ± 3067	48434 ± 22279	33100 ± 7214	21028 ± 3276	24016 ± 4874
Unidentified sesquiterpenoid 5	4412 ± 1822	21622 ± 5700	22486 ± 2478	25962 ± 5800	43914 ± 25214	31228 ± 6477	103152 ± 43793	71754 ± 14078	49452 ± 6913	54393 ± 11046
Unidentified sesquiterpenoid 6	173 ± 23	465 ± 130	2854 ± 896	1652 ± 595	3560 ± 1022	2947 ± 1685	16799 ± 7467	7454 ± 1432	13697 ± 3104	5838 ± 1266
Unidentified sesquiterpenoid 7	139 ± 41	695 ± 109	1948 ± 328	1588 ± 584	4096 ± 1747	2662 ± 1581	14001 ± 5710	9098 ± 1809	6285 ± 2205	5892 ± 1303
Unidentified sesquiterpenoid 8	382 ± 253	3059 ± 1203	2998 ± 653	2416 ± 853	56459 ± 54709	5151 ± 2455	5419 ± 1254	5515 ± 1318	15283 ± 10122	5126 ± 1943
Unidentified sesquiterpenoid 9	73 ± 15	2822 ± 1123	2573 ± 682	4456 ± 1158	58192 ± 54134	4378 ± 2612	5871 ± 2164	6677 ± 2495	14806 ± 10156	6111 ± 1746
Unidentified sesquiterpenoid 10	117 ± 15	1186 ± 534	2482 ± 880	2338 ± 850	252935 ± 250576	4748 ± 3304	13830 ± 6033	7531 ± 1544	5445 ± 1303	7044 ± 1875
Unidentified sesquiterpenoid 11	322 ± 70	2750 ± 892	6403 ± 1945	5554 ± 1741	6034 ± 2362	11258 ± 7292	34463 ± 15281	18395 ± 3512	15729 ± 3691	16569 ± 3698
Unidentified sesquiterpenoid 12	224 ± 39	2302 ± 1061	5364 ± 2241	5242 ± 2043	5065 ± 2782	10328 ± 7584	28834 ± 17085	15821 ± 3654	10415 ± 5457	14867 ± 4506
Unidentified sesquiterpenoid 13	121 ± 27	263 ± 81	1215 ± 427	535 ± 135	1052611 ± 1051907	830 ± 118	1757 ± 644	637 ± 126	1787 ± 541	1287 ± 420
Unidentified sesquiterpenoid 14	219 ± 57	231 ± 63	565 ± 214	483 ± 133	577 ± 146	835 ± 497	2180 ± 935	1382 ± 312	1404 ± 426	1109 ± 226
Unidentified sesquiterpenoid 15	242 ± 68	273 ± 66	324 ± 56	471 ± 94	534 ± 191	363 ± 151	868 ± 331	770 ± 141	924 ± 410	498 ± 170
Aromatic compounds										
Benzaldehyde*	417 ± 100	194 ± 48	1304 ± 684	206 ± 34	769 ± 252	255 ± 37	336 ± 61	199 ± 62	854 ± 240	850 ± 488
Benzyl alcohol	552 ± 136	655 ± 153	47767 ± 21358	1071 ± 287	238749 ± 237403	3164 ± 2315	99719 ± 98185	31851 ± 17978	81029 ± 29980	1591 ± 569
Salicylaldehyde*	292 ± 167	420 ± 348	8081 ± 6875	102 ± 36	1619 ± 1068	156 ± 80	5641 ± 2656	135 ± 20	221698 ± 217627	185 ± 61
1-Phenylethanone	601 ± 214	304 ± 104	2374 ± 1335	282 ± 63	1976 ± 1044	301 ± 45	587 ± 111	401 ± 59	1329 ± 787	461 ± 140
2-Methoxyphenol	236 ± 59	191 ± 43	808 ± 269	224 ± 55	1418 ± 872	307 ± 107	562 ± 40	207 ± 89	1086 ± 202	463 ± 126
2-Phenylethanol	319 ± 155	596 ± 124	2103 ± 768	612 ± 133	5450 ± 3643	725 ± 175	3834 ± 1864	1108 ± 243	3022 ± 519	1870 ± 658
Methyl salicylate	266 ± 100	4080 ± 2474	298 ± 78	216 ± 129	184 ± 58	89 ± 21	379 ± 81	228 ± 102	1389 ± 440	192 ± 85
Eugenol*	114 ± 50	46 ± 7	16976 ± 16677	105 ± 49	995 ± 711	150 ± 76	278 ± 153	51 ± 4	22140 ± 20285	215 ± 127
Benzoic acid- *n*-pentyl ester	139 ± 36	1135 ± 227	1516 ± 475	1304 ± 230	2422 ± 956	1493 ± 383	9501 ± 3839	5103 ± 1013	3492 ± 832	4123 ± 740
2-Phenylpropan-2-ol	10034 ± 2859	25462 ± 5010	24642 ± 3516	42482 ± 10202	64247 ± 32289	30372 ± 2976	83187 ± 15863	53856 ± 12000	50873 ± 6616	71991 ± 29825

	Control (no herbivore)		*Lymantria dispar*		*Laothoe populi*		*Phratora vulgatissima*		*Chrysomela populi*	
	control for damaged leaves	control for adj. undamaged leaves	damaged leaves	adjacent undamaged leaves	damaged leaves	adjacent undamaged leaves	damaged leaves	adjacent undamaged leaves	damaged leaves	adjacent undamaged leaves
Compound	**mean ± SE**	**mean ± SE**	**mean ± SE**	**mean ± SE**	**mean ± SE**	**mean ± SE**	**mean ± SE**	**mean ± SE**	**mean ± SE**	**mean ± SE**
Nitrogenous compounds										
2-Methylbutyraldoxime*	773 ± 618	456 ± 185	17406 ± 10139	595 ± 232	22099 ± 14042	469 ± 80	24609 ± 4231	255 ± 36	34642 ± 7792	1945 ± 1428
3-Methylbutyraldoxime*	2283 ± 526	692 ± 272	4222 ± 1156	679 ± 173	3014 ± 1115	748 ± 306	914 ± 356	563 ± 317	1789 ± 515	1094 ± 255
Methoxy phenyloxime	52885 ± 32679	27546 ± 13920	63928 ± 32245	32211 ± 12947	210029 ± 111149	47280 ± 15113	63393 ± 33543	32296 ± 15592	75678 ± 30192	25813 ± 14517
1-Methyl-2-pyrrolidinone	204 ± 39	2242 ± 2171	1472 ± 424	4339 ± 2597	1136 ± 703	747 ± 426	422 ± 53	70 ± 16	2983 ± 1340	4358 ± 2779
Benzyl-cyanide*	6002 ± 5897	19627 ± 12486	167202 ± 162887	2594 ± 1879	59559 ± 25678	12000 ± 6077	288693 ± 98010	1529 ± 1324	586122 ± 196588	10521 ± 10343
2-phenylnitroethane*	574 ± 96	387 ± 70	1277 ± 640	556 ± 241	1980 ± 1320	343 ± 57	472 ± 110	266 ± 47	1421 ± 439	326 ± 133
N-phenylaniline	307 ± 75	41 ± 16	634 ± 387	67 ± 20	285 ± 113	100 ± 33	177 ± 63	35 ± 11	257 ± 120	76 ± 16
Unidentified nitrogenous	1331 ± 224	1135 ± 487	10102 ± 4760	5741 ± 3590	12372 ± 7265	3945 ± 1728	7406 ± 3527	2779 ± 2347	80405 ± 58354	6224 ± 3498
Indole*	173 ± 35	74 ± 33	1148 ± 929	106 ± 32	564 ± 288	69 ± 9	20286 ± 3785	70 ± 10	24022 ± 7726	481 ± 364
Green Leaf Volatiles										
Hexan-1-ol	906 ± 182	500 ± 285	2837 ± 1496	670 ± 204	976 ± 242	400 ± 111	803 ± 293	275 ± 82	2497 ± 1020	755 ± 363
2-Hexenal	15932 ± 13856	421 ± 110	6657 ± 3251	360 ± 106	27762 ± 22810	717 ± 459	4523 ± 969	233 ± 45	7663 ± 2305	4897 ± 4238
3-Hexenal	1042 ± 396	104 ± 33	3058 ± 1477	160 ± 65	50561 ± 49689	139 ± 62	1403 ± 687	101 ± 57	14918 ± 8341	255 ± 158
3-Hexenol*	17845 ± 15507	165 ± 77	61508 ± 50904	521 ± 242	72640 ± 43962	542 ± 205	5095 ± 3313	334 ± 163	21429 ± 12880	410 ± 66
2-Hexenol	1105 ± 404	804 ± 427	2476 ± 1002	263 ± 124	7555 ± 4196	506 ± 196	2916 ± 1039	981 ± 329	8662 ± 2083	952 ± 602
3-Hexenyl acetate*	4052 ± 1274	7605 ± 2849	7737 ± 580	13022 ± 3006	9875 ± 2925	11934 ± 2499	14508 ± 2035	17341 ± 2686	7310 ± 1633	31251 ± 12879
Hexyl acetate	2233 ± 417	3089 ± 587	6748 ± 2208	4420 ± 895	4920 ± 828	3873 ± 863	5338 ± 712	5227 ± 736	6009 ± 1605	7111 ± 1571
2-Hexenyl acetate*	2187 ± 1105	471 ± 101	3062 ± 725	497 ± 126	3900 ± 2278	657 ± 162	1734 ± 496	742 ± 136	5548 ± 2705	1186 ± 422
3-Hexenyl isobutyrate*	1618 ± 451	3329 ± 630	4476 ± 1112	5967 ± 1449	7754 ± 3850	4002 ± 383	10362 ± 1966	6891 ± 1493	5603 ± 919	9252 ± 3781
3-Hexenyl butyrate	1739 ± 369	1075 ± 376	4742 ± 2902	1436 ± 526	4838 ± 3133	1650 ± 639	2191 ± 433	863 ± 345	5489 ± 2120	1857 ± 1018
3-Hexenyl benzoate*	1221 ± 400	2586 ± 894	3066 ± 554	4287 ± 1575	6891 ± 4794	6158 ± 3075	3583 ± 1032	11774 ± 7296	3240 ± 585	7857 ± 4423
3-Hexenyl 2-methyl butanoate	114 ± 21	192 ± 36	1064 ± 411	507 ± 183	1896 ± 1200	539 ± 177	2199 ± 522	853 ± 336	2058 ± 503	817 ± 262
Other										
Heptane	133820 ± 40375	92514 ± 16179	448936 ± 149269	276418 ± 198043	378177 ± 175996	33453 ± 16577	149208 ± 47230	52284 ± 17519	374848 ± 76138	2839497 ± 2774479
Oxabicyclo-hexan-2-one	142 ± 16	177 ± 103	616 ± 171	98 ± 24	974 ± 551	118 ± 18	340 ± 70	71 ± 22	337 ± 125	201 ± 49
Hydroxyethoxyethanol	2471 ± 472	807 ± 290	3528 ± 1215	2021 ± 718	4959 ± 2023	1168 ± 212	2527 ± 371	641 ± 144	3994 ± 750	629 ± 117
Methylheptenone	504 ± 323	92 ± 20	481 ± 247	164 ± 59	4820 ± 4594	115 ± 23	143 ± 22	92 ± 7	457 ± 153	262 ± 106
2-Hydroxycyclohexanone	1157 ± 172	852 ± 302	1384 ± 366	1271 ± 588	1316 ± 745	975 ± 301	475 ± 192	1257 ± 446	2420 ± 1588	878 ± 489
1.2-Cyclohexanediol	226 ± 51	208 ± 66	1435 ± 711	384 ± 125	15339 ± 10592	606 ± 388	4430 ± 1363	140 ± 74	5685 ± 3360	784 ± 235
Nonanal*	4088 ± 2373	2494 ± 1728	15045 ± 11384	3067 ± 1905	3500 ± 2529	1368 ± 631	1482 ± 895	178 ± 33	2674 ± 1259	4434 ± 3103
Decanal	3463 ± 1625	2647 ± 1067	7432 ± 2709	2861 ± 1689	336133 ± 314834	1399 ± 621	19393 ± 13568	427 ± 193	61014 ± 25721	1598 ± 794
β-Cyclocitral	933 ± 204	537 ± 271	1451 ± 518	634 ± 238	995 ± 389	379 ± 179	247 ± 59	355 ± 144	748 ± 335	1068 ± 436
Nonanoic acid	8919 ± 1065	3336 ± 1677	23322 ± 9515	3336 ± 1202	11011 ± 2385	2774 ± 1578	7092 ± 2692	981 ± 533	15462 ± 9147	4267 ± 2395
Undecanal	288 ± 57	203 ± 48	2260 ± 1468	514 ± 180	1122 ± 550	195 ± 56	546 ± 227	177 ± 24	1033 ± 232	599 ± 250
Octylether	942 ± 135	441 ± 133	1428 ± 603	759 ± 148	10253 ± 8101	625 ± 171	1387 ± 276	972 ± 446	3908 ± 1038	1043 ± 346
Jasmone	5317 ± 1805	29915 ± 7504	30665 ± 3306	35189 ± 7598	59918 ± 34714	41967 ± 8444	130653 ± 53135	92114 ± 17122	65349 ± 9043	70956 ± 14125

* Confirmed by comparison of retention time and mass spectrum to that of internal standard (source of standard shown in table S2).

(a) compounds were identified by ≥ 95% similarity to structures in WILEY8 or NIST databases.

Table S2 Internal standards used for the identification of volatile organic compounds in black poplar

Standard	CAS-Nr.	Source
(*E*)-2-Hexenyl acetate	2497-18-9	Sigma-Aldrich (Taufkirchen, Germany)
(*Z*)-3-Hexen-1-ol	928-96-1	Bedoukian (Danbury, CT, USA)
(*Z*)-3-Hexenyl benzoate	3681-71-8	Sigma-Aldrich (Taufkirchen, Germany)
(*Z*)-3-Hexenyl acteate	3681-71-8	Sigma-Aldrich (Taufkirchen, Germany)
(*Z*)-3-Hexenyl isobutyrate	41519-23-7	Sigma-Aldrich (Taufkirchen, Germany)
2-Methylbutyraldoxime (*E:Z*, 3:1)	49805-56-3	Chemical synthesis (Irmisch *et al*. (2013)
2-Phenylnitroethane	6125-24-2	Apin Chemicals (Abingdon, UK)
3-Methylbutyraldoxime (*E:Z*, 2:1)	626-90-4	Chemical synthesis (Irmisch *et al*. (2013)
Benzaldehyde	100-52-7	Fluka (Munich, Germany)
Benzyl cyanide	140-29-4	Sigma-Aldrich (Taufkirchen, Germany)
Borneol	464-45-9	Fluka (Munich, Germany)
Camphor	24368-68-3	Fluka (Munich, Germany)
1,8-Cineole	470-82-6	Sigma-Aldrich (Taufkirchen, Germany)
Eugenol	97-53-0	Sigma-Aldrich (Taufkirchen, Germany)
Indole	120-72-9	Sigma-Aldrich (Taufkirchen, Germany)
Linalool	78-70-6	Sigma-Aldrich (Taufkirchen, Germany)
Linalool oxide	60047-17-8	Fluka (Munich, Germany)
Nerolidol	7212-44-4	Sigma-Aldrich (Taufkirchen, Germany)
Nonanal	124-19-6	Sigma-Aldrich (Taufkirchen, Germany)
Salicylaldehyde	90-02-8	Acros Organics (Thermo Fischer Scientific, Geel, Belgium)
Terpinen-4-ol	562-74-3	Thermo Fischer Scientific (Geel, Belgium)
α-Humulene	6753-98-6	Fluka (Munich, Germany)
α-pinene	7785-26-4	Fluka (Munich, Germany)
α-terpinene	99-86-5	Fluka (Munich, Germany)
α-Terpineol	10482-56-1	Sigma-Aldrich (Taufkirchen, Germany)
(*E*)-ß-ocimene	3338-55-4	Chemos (Regenstauf, Germany)

Reference:

Irmisch S et al. (2013) Two herbivore-induced cytochrome P450 enzymes CYP79d6 and CYP79d7 catalyze the formation of volatile aldoximes involved in poplar defense. Plant Cell 25:4737-4754.doi:10.1105/tpc.113.118265

10.2 MANUSCRIPT II – SUPPLEMENTARY DATA

Supplementary Material:

Title: Poplar protease inhibitor expression differs in an herbivore specific manner

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Table S1. Feeding damage on black poplar leaves after 2 d of herbivore feeding by *Lymantria dispar*, *Amata mogadorensis* and *Phratora vulgatissima* and trypsin-inhibiting activity in these leaves. Shown is the leaf area loss (% of total leaf area) and inhibitory activity (equivalents to soybean trypsin inhibitor (STI), µg STI g^{-1} dry weight (DW)) as mean ± SEM (n = 6). The three groups were not found to be significantly different (ANOVA: $F_{(2)}$ = 0.762; P = 0.484). Data are taken from Fabisch *et al.* (2019), and the parameters re-calculated for the six replicates selected for this study.

Herbivore	**Damage (%)**	**KTI activity (µg g^{-1} DW)**
L. dispar	19.0 ± 5.7	95.7 ± 12.9
A. mogadorensis	12.3 ± 4.8	93.1 ± 26.4
P. vulgatissima	12.0 ± 1.6	257.6 ± 23.2

Table S2. Sequences of primers used for full-length cloning (FL) and gene expression analysis via quantitative real-time PCR (qRT-PCR). Primer sequences for *Actin* were taken from Ramiraz-Carvajal*et al.* 2008[1].

Usage	Name	Forward primer (5' → 3')	Reverse primer (5' → 3')
FL	SQ16949	ATGAAGATCACTAACGTTCTAG	CTATTCATCTGGTTCAATCATAAC
FL	SQ33325 (a)*	CACCCATGTGTCTGATGTGG	TCAATATGCATCTGGTTCG
	SQ33325 (b)*	ATGGAGATCACTAAATTTCTAGG	GTATCCTTCCGTTGTTGGACT
FL	SQ33325-2	ATGGAGATCACTAAATTTCTAGG	TCAATATGCATCTGGTTCG
FL	SQ14956	ATGAAGATCACTAAATTTCTAGGG	TTATGTGCTCTCAATGCG
FL	SQ36485	ATGAAGATCACCAAGTTTGT	TCAGGAGCTTTTATCTGC
FL	SQ2215	ATGAAGATCTCTAACTTTCTAGTG	TTACACCATTTTATACTCTATTTTAGA
FL	SQ1996	ATGAAGATTACTAACTTTCTAGTGC	TTACATCATTTTATACTCTATTTTAGAAGA
FL	VP33729	ATGAAGTCTACATTGTTGGT	TCATATGGATGAACTTAAAGGC
FL	SQ6530	ATGAAGTCTACATTGTTGGT	TCATATGGATGAACTTAAAGGC
FL	SQ287	ATGAAGAATATTATGTTACTACCCC	TTACACAACAGCTTTTAATCC
qRT-PCR	SQ2215	TTACTGTCTCCAATGAGCCATG	ATGAGCAGATGGGTTCGG
qRT-PCR	SQ287	GCGTTACAGGTACACCAG	ACAAATGAGCCTCCCACG
qRT-PCR	SQ36485	ACAACTCTTGCGGTCTCTG	CATTGTTGGCCTCAACTCC
qRT-PCR	SQ6530	TTCGGACCTGTTACAAGGC	GAAACTAGCTTGTACCCTATGC
qRT-PCR	SQ1580	GTTGTGTTTTCTCCAATGAGCG	GGACGCATGAGCATTCACAT
qRT-PCR	VP33729	CTAATATGCCAGCCTTCTC	GTTGGACCAGTTACAAGGT
qRT-PCR	SQ34879	CACCCATGTGTCTGATGTG	GAAGAGGGCCGACATTGG
qRT-PCR	SQ8996	GCGCAACCGGTTTAAACC	GATTCAATTCAACAGCCAAAG
qRT-PCR	Actin	CCCATTGAGCACGGTATTGT	TACGACCACTGGCATACAGG

* two primer pairs were used to amplify the gene in two parts. [1] Ramirez-Carvajal GA, Morse AM, Davis JM. 2008. *New Phytologist* **177:**77–89.

Table S3. Contigs in the transcriptome of black poplar leaves damaged by *L. dispar* and *P. vulgatissima* that were annotated as protease inhibitors, and their differential expression (ratio of RPKM values) compared to the respective undamaged control treatments (Ctrl 1 and 2). Contigs are sorted by their regulation pattern (up-regulated, differentially regulated, down-regulated upon herbivory) and further by the *P*-value of 'Ctrl 1 vs. *L. dispar*' in descending significance. Annotations including 'Kunitz' are marked in bold; for these contigs, the gene names used in this study are given (PnKTI).

Name			**Ctrl 1 vs. *L. dispar***		**Ctrl 2 vs. *P. vulgatissima***	
ID	**PnKTI**	**Sequence Annotation**	**Diff. expr.**	***P***	**Diff. expr.**	***P***
Up-regulated upon herbivory						
SQ1996	*PnKTI A6*	**Kunitz trypsin inhibitor ti3**	4.511 up	0.00193	7.782 up	0.0000667
SQ287	*PnKTI D2*	**Kunitz trypsin inhibitor**	1547.915 up	0.00318	251.880 up	0.00045
SQ14956	*PnKTI A14*	**Kunitz trypsin inhibitor**	1340.240 up	0.00828	3121.864 up	0.0000843
SQ6530	*PnKTI B5*	**Kunitz trypsin inhibitor**	139.616 up	0.00852	127.805 up	0.00505
SQ36485	*PnKTI A7*	**Kunitz-type protease inhibitor kpi-**	1512.845 up	0.00916	2087.716 up	0.0000093
SQ27194	*PnKTI A12*	**Kunitz trypsin inhibitor 3**	12.698 up	0.0105	2.847 up	0.0288
SQ8430		inter-alpha-trypsin inhibitor heavy	5.710 up	0.0138	3.448 up	0.0242
SQ16949	*PnKTI A2*	**Kunitz-type protease inhibitor kpi-**	1609.628 up	0.0141	2529.060 up	0.0000375
SQ37196		inter-alpha-trypsin inhibitor heavy	5.611 up	0.0146	3.428 up	0.0113
SQ17376		inter-alpha-trypsin inhibitor heavy	6.847 up	0.0152	4.168 up	0.012
SQ8431		inter-alpha-trypsin inhibitor heavy	4.228 up	0.0165	2.870 up	0.00411
SQ24859		inter-alpha-trypsin inhibitor heavy	5.591 up	0.0218	2.972 up	0.0166
SQ2215	*PnKTI A15*	**Kunitz trypsin inhibitor ti3**	40.816 up	0.0229	72.350 up	0.000316
SQ33325	*PnKTI A13*	**Kunitz-type protease inhibitor KPI-**	24381.159 up	0.0247	7431.743 up	0.00283
SQ49470		inter-alpha-trypsin inhibitor heavy	8.483 up	0.0832	4.155 up	0.065
SQ24955	*PnKTI B2*	**Kunitz trypsin protein inhibitor 3**	38.630 up	0.188	3.239 up	0.277
SQ43060	*PnKTI C1*	**truncated Kunitz trypsin inhibitor family protein**	40.465 up	0.192	58.511 up	0.0764
SQ6918		protease inhibitor seed storage lipid transfer family protein	2.266 up	0.227	1.814 up	0.00185
SQ61412		protease inhibitor seed storage lipid transfer family protein	14.550 up	0.392	153.666 up	0.0619
VP33729	*PnKTI B1*	**Kunitz trypsin inhibitor 4**	114,626 up	0,409	na	na
SQ47062		inter-alpha-trypsin inhibitor heavy	2.573 up	0.441	2.334 up	0.584
SQ49510	*PnKTI C3*	**truncated Kunitz trypsin inhibitor family protein**	14.149 up	0.458	40.486 up	0.228
SQ22824		protease inhibitor seed storage lipid transfer family protein	7.775 up	0.489	1.674 up	0.344
SQ10660		protease inhibitor seed storage lipid transfer family protein	1.874 up	0.507	1.953 up	0.178

Name			Ctrl 1 vs. *L. dispar*		Ctrl 2 vs. *P. vulgatissima*	
ID	**PnKTI**	**Sequence Annotation**	**Diff. expr.**	***P***	**Diff. expr.**	***P***
SQ23804	*PnKTI C6*	**truncated Kunitz trypsin inhibitor family protein**	7.688 up	0.549	3.869 up	0.735
SQ172	*PnKTI C7*	**truncated Kunitz trypsin inhibitor family protein**	1.778 up	0.552	12.017 up	0.0805
SQ56957	*PnKTI C5*	**truncated Kunitz trypsin inhibitor family protein**	4.550 up	0.747	3.794 up	0.765
SQ21088		protease inhibitor seed storage lipid transfer family protein	1.224 up	0.875	1.398 up	0.539
SQ33797		serine protease inhibitor	1.229 up	1	3.318 up	0.785
Differentially regulated upon herbivory						
SQ29472		serine-type endopeptidase inhibitor	108.536 down	0.00000369	4.379 up	0.756
SQ47318		cysteine proteinase inhibitor 12-like	1.434 down	0.205	1.439 up	0.633
SQ10062		cysteine protease inhibitor	1.306 up	0.324	1.004 down	1
SQ26391		protease inhibitor seed storage lipid transfer family protein	1.527 up	0.858	1.583 down	0.608
SQ17957		inter-alpha-trypsin inhibitor heavy chain-related family protein	1.090 down	0.911	1.231 down	0.658
SQ12658		cysteine proteinase inhibitor	1.001 up	1	1.176 down	0.756
SQ18761		cysteine proteinase inhibitor	1.046 up	1	1.031 down	1
Down-regulated upon herbivory						
SQ64031		cysteine proteinase inhibitor b-like	5.168 down	0.0282	3.143 down	0.0767
SQ7576		cysteine proteinase inhibitor 12-like	1.318 down	0.0655	1.923 down	0.103
SQ35734		subtilisin inhibitor	1.744 down	0.142	2.001 down	0.168
SQ7450		cysteine proteinase inhibitor	1.596 down	0.22	1.370 down	0.214
SQ52953		cysteine proteinase inhibitor 12-like	1.232 down	0.509	1.259 down	0.663
SQ7577		cysteine inhibitor 1	1.140 down	0.6	1.262 down	0.174
SQ34850		inhibitor of trypsin and hageman factor-like protein	1.710 down	0.715	1.666 down	0.658
SQ17958		inter-alpha-trypsin inhibitor heavy chain-related family protein	1.058 down	0.851	1.095 down	0.68

na - not available; this contig and its expression was taken from another transcriptome of the same *P. nigra* genotype (unpublished) with comparable *L. dispar*, but not *P. vulgatissima* herbivory treatment.

Table S4. Nomenclature of Kunitz-type protease inhibitors reported in this study and their corresponding homologs as Potri-IDs from the *Populus trichocarpa* genome v3.0 and genes described in other studies. Similarity values (% sim) show the percentage of identical base pairs between the respective sequences with those published in our study based on their full-length open reading frame, unless stated otherwise.

Eberl *et al.* 2020	***P. trichocarpa* genome**		**Ma *et al.* 2011** [1]		**Philippe *et al.* 2009** [2]		**Other studies**	
Name (*P. nigra*)	**Potri-ID**	**% sim**	**Name (*P. nigra*)**	**% sim**	**Name (*P. spp*)**	**% sim**	**Name (*P. spp*)**	**% sim**
PnKTI A2	Potri.010G007800.1	98.5	-	-	PtxnKPI-A2	98.5	TI6[3]	97.7
PnKTI A4	Potri.010G007900.1	99.3	-	-	PtxnKPI-A5/ PtxdKPI-A5	99.3/ 99.2		
PnKTI A6	Potri.019G124400.1	97.7	PnKTI A6	98.0	PtxnKPI-C6.1/ PtxdKPI-C7*	99.5/ 99.5	TI3[3]	98.0
PnKTI A7	Potri.019G121900.1	100	PnKTI A7	99.5	PtxdKPI-C2.1	99.5		
PnKTI A12	Potri.003G097900.2	100	PnKTI A12	99.2	PtiKPI-2	99.2		
PnKTI A13	-	-	-	-	-	-		
PnKTI A14	Potri.T029200.1*	92.2	-	-	PtxdKPI-B5*/ PtxnKPI-B7*	98.3/ 97.1	GWIN 3[4]; PnTIH1.1[5]	98.5/100
PnKTI A15	-	-	-	-	-	-		
PnKTI B1	Potri.004G067800.1	99.7	PnKTI B1	99.7	PtiKPI-D1.2	99.7	TI4[3]	99.7
PnKTI B2	Potri.004G067900.1	99.1	PnKTI B2	98.9	PtxdKPI-D2	99.1		
PnKTI B5	-	-	-	-	PtxnKPI-D8	100		
PnKTI C1	Potri.001G309900.1	97.2	PnKTI C1	99.7	-	-		
PnKTI C3	Potri.007G111600.1	99.2	PnKTI C3	99.2	-	-		
PnKTI C5	Potri.004G000400.1	99.2	PnKTI C5	99.2	PtxnKPI-F4	99.3		
PnKTI C6	Potri.019G011000.1	98.8	PnKTI C6	100	-	-		
PnKTI C7	Potri.007G111800.1	96.6	PnKTI C7	96.7	PtxdKPI-F9	97.4		
PnKTI D2	Potri.019G088200.1	97.9	PnKTI D2*	99.2	PtxdKPI-E1	99.4	TI5[4]	99.2

[1] Ma Y, Zhao Q, Lu M-Z, Wang J (2011). *Tree Genetics & Genomes*, **7:** 431-441.[2] Philippe RN, Ralph SG, Külheim C, Jancsik SI, Bohlmann J (2009). *New Phytologist*, **184:** 865-884. [3] Major IT &Constabel CP (2008). *Plant Physiology*, **146:** 888-903.[4]Bradshaw HD, Hollick JB, Parsons TJ, Clarke HRG, Gordon MP. *Plant Molecular Biology*, **14:** 51-59. [5] Nishiguchi M, Yoshida K, Sumizono T, Tazaki K (2002). *Molecular Genetics and Genomics*, **4:** 506-514. * incomplete ORF.

Table S5. C_q (quantification cycle) values of the qRT-PCR analysis for individual *KTI* genes in black poplar leaves without damage (Control) or after herbivory by lepidopteran caterpillars (*L. dispar, A. mogadorensis*) or adult beetles (*P. vulgatissima*). Shown are means ± SEM for each treatment group (n = 6).

Gene	Control	*L. dispar*	*A. mogadorensis*	*P. vulgatissima*
PnKTI A6	23 ± 0.3	19 ± 0.4	19 ± 0.7	17 ± 0.3
PnKTI A7	31 ± 0.6	27 ± 1.0	28 ± 0.6	26 ± 0.5
PnKTI A13	31 ± 0.5	20 ± 0.8	21 ± 0.8	17 ± 0.4
PnKTI A14	27 ± 0.5	17 ± 0.8	17 ± 0.7	14 ± 0.4
PnKTI A15	25 ± 0.3	18 ± 0.5	18 ± 0.5	16 ± 0.2
PnKTI B1	33 ± 0.7	27 ± 0.8	27 ± 0.8	23 ± 0.8
PnKTI B5	36 ± 0.9	32 ± 0.6	31 ± 0.7	28 ± 0.5
PnKTI D2	35 ± 0.4	25 ± 0.8	23 ± 0.8	20 ± 0.6
Actin*	20 ± 0.4	20 ± 0.2	20 ± 0.4	20 ± 0.2

* house-keeping gene for normalization

10.3 MANUSCRIPT III – SUPPLEMENTARY DATA

Supplemental material

Title: Volatile mediated defense priming in black poplar. Minor changes can cause major differences

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Qualification and quantification of volatile organic compounds

2 µl of the eluate was injected splitless into a gas chromatograph (6890 series, Hewlett-Packard, Agilent Technologies, Santa Clara, CA, USA) equipped with a 30 m × 250 µm × 0.25 µm DB5-MS column (WicomGmbH, Heppenheim, Germany) coupled to a quadrupole mass spectrometer (5973 series, Hewlett-Packard, Agilent Technologies, Santa Clara, CA, USA) (in short GC/MS). The injector was held at 230 °C with helium used as carrier gas at 1 ml/min. The oven temperature of the GC/MS was held at 50 °C for 3 minutes after injection and then heated up to 95 °C at a rate of 4 °C/min. Afterwards, the oven temperature was increased to 145 °C with a 15 °C/min gradient and then to 180 °C with a 10 °C/min gradient. Finally, the oven temperature was kept stable for 3 min at 300 °C. Mass spectra were recorded (transfer line temperature: 230 °C, source temperature: 230 °C, quadrupole temperature: 150 °C, ionization energy: 70 eV, mass range: 40-500 m/z). Compounds were identified by comparing their mass spectra to authentic standards and three libraries (Wiley275, NIST, ADAMS). For quantification, the samples were separated with the same GC method as described above with hydrogen as the carrier gas. Afterwards the samples were analyzed with a flame ionization detector (FID, 9200 Hydrogen detector, Packard, Agilent Technologies, Santa Clara, CA, USA) operating at 300 °C. Absolute amounts of all compounds were calculated based on the relation of their FID peak area and the area of the internal standard according to the “effective carbon number (ECN) concept” (Scanlon and Willis 1985).

Table S1: Means ± Standard Error (SE) of constitutive or herbivore induced volatile emissions from black poplar emitter trees (*n* = 4) in ng g^{-1} (fw) h^{-1}.

	constitutive		Herbivory induced		U-test	
	Mean	±SE	Mean	±SE	U	p
GLV	**96.14**	**32.12**	**1228.35**	**532.50**	**2.309**	**0.021**
Z-3 hexenol	3.65	1.32	266.56	129.92	**2.309**	**0.021**
Z-3-hexenylacetate	92.50	31.08	961.79	457.22	2.309	0.021
MT	**355.96**	**129.92**	**2951.04**	**1607.43**	**2.309**	**0.021**
α-pinene	24.93	7.25	55.45	24.14	ns	ns
camphene	39.42	11.95	76.55	33.39	ns	ns
sabinene	30.28	10.44	59.88	31.20	ns	ns
β-pinene	11.95	9.77	6.00	3.76	ns	ns
myrcene	24.99	6.73	59.22	33.97	ns	ns
limonene	72.10	43.35	150.37	127.81	ns	ns
Z-ocimene	18.94	8.34	209.92	115.72	2.309	0.021
E-β-ocimene	100.75	37.58	2246.59	1200.03	2.309	0.021
camphor	20.62	10.67	64.46	29.30	ns	ns
borneol	11.98	5.28	22.61	11.94	ns	ns
***E*-DMNT**	**18.27**	**7.30**	**1379.98**	**678.91**	**2.309**	**0.021**
ST	**157.02**	**88.81**	**616.77**	**355.08**		
β-caryophyllene	13.73	8.91	248.72	140.95	2.309	0.021
α-humulene	9.37	6.14	50.28	31.84	1.732	0.083
β-cubebene	7.80	4.73	133.07	76.76	2.323	0.02
E,*E*-α-farnesene	120.39	67.49	170.98	97.06	ns	ns
δ-cadinene	5.73	2.53	13.72	8.53	ns	ns
Aromatics	**31.94**	**16.53**	**248.80**	**114.11**	**1.732**	**0.083**
benzaldehyde	8.22	2.19	17.52	6.77		
salicylaldehyde	0.00	0.00	199.13	97.14	2.460	0.014
eugenol	23.72	14.56	24.05	12.51	ns	ns
Nitrogenous	**0.00**	**0.00**	**433.81**	**185.99**	**2.460**	**0.014**
E-2-methylbutyraldoxime	0.00	0.00	223.59	97.50	1.984	0.047
Z-2-methylbutyraldoxime	0.00	0.00	63.47	23.47	2.460	0.014
(*E*/*Z*)-3-methylbutyraldoxime	0.00	0.00	63.22	20.79	2.460	0.014
benzyl alcohol	0.00	0.00	8.09	2.72	1.984	0.047
benzyl cyanid	0.00	0.00	83.53	54.59	1.984	0.047
isoamylacetate	**0.00**	**0.00**	**93.48**	**46.27**	**2.460**	**0.014**

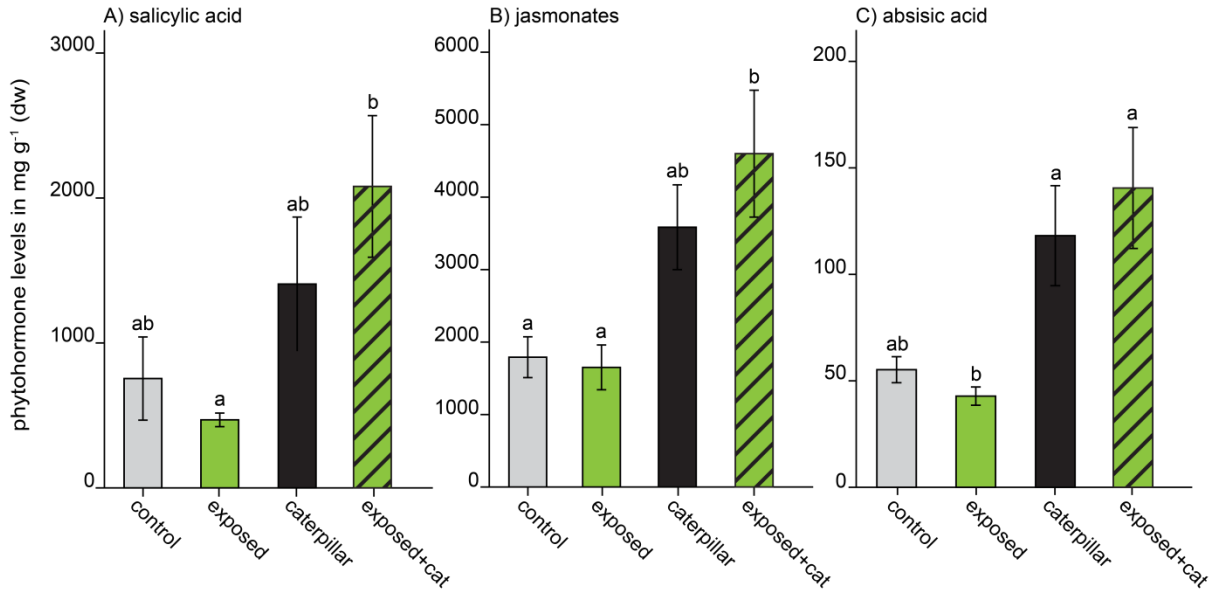

Figure S1: Salicylic acid (A), jasmonates (B) and absisic acid (C) concentrations of black poplar leaves after herbivore damage by gypsy moth caterpillars (caterpillar), previous exposure to HIPVs (HIPV exposed) and a combination of herbivore damaged and previously exposed to HIPVs (HIPV exposed + caterpillar) compared to control plants (control). The group jasmonates represents the sum of jasmonic acid and its derivatives (JA-Ile, OH-JA, OH-JA-Ile, COOH-JA-Ile and cisOPDA). Different letters indicate significant differences between treatments based on a Kruskal Wallis test with Dunns post hoc (A: H = 11.894; p = 0.008; B: H = 15.123; p = 0.002; C: H = 15.663; p = 0.001). Bars represent means ± SE; n = 5-6.

RNA isolation, RNA-Seq, de novo assembly and differential gene expression analysis

RNA was isolated from frozen, ground leaf material using the InviTrap Spin Plant Mini Kit (Stratec Biomedical AG) according to the manufacturer's manual. Additionally, a DNA digestion was included (DNase set; Qiagen). RNA concentration and purity were tested with a NanoDrop2000c spectrophotometer (Peqlab Biotechnology AG).

Sequencing of the poly(A)+ mRNA enriched samples was done at the Max Planck-Genome-Centre (Köln, Germany) on a HighSeq3000 instrument (Illumina, San Diego, California, USA) generating appr. 15 Mio paired-end reads (2 x 150 bp) per sample. Quality control measures, including the filtering of high-quality reads based on fastq file scores, the removal of reads containing primer/adapter sequences, and trimming of the read length, were carried out using CLC Genomics Workbench v11 (http://www.clcbio.com). The same software was used for *de novo* transcriptome assembly using a total of 185 Mio sequence reads, combining three replicates of each RNA-Seq treatment group, and selecting the presumed optimal consensus transcriptome as previously described (Vogel et al. 2014). The final *de novo* reference transcriptome assembly (backbone) of *Populus nigra* contained 65,866 contigs. Minimum contig size was 300 bp with an N50 contig size of 1430 bp. The transcriptome was annotated using BLAST, Gene Ontology (GO) and InterPro terms (InterProScan, EBI), enzyme classification (EC) codes, and metabolic pathways (Kyoto Encyclopedia of Genes and Genomes, KEGG) as implemented in BLAST2GO v5.1 (http://www.blast2go.de). To assess transcriptome completeness, we performed a BUSCO (Benchmarking Universal Single-Copy Orthologs; http://busco.ezlab.org) analysis by comparing our assembled transcript set against a set of highly conserved single-copy orthologs. This was accomplished using the BUSCO v3 pipeline (Waterhouse et al. 2017) compared to the predefined set of 303 Eukaryota single-copy orthologs from the OrthoDB v9.1 database. The assembled *P. nigra* transcriptome was determined to be 82.2% complete and 6.2% of the BUSCO genes were missing. The Illumina data have been deposited in the EBI short read archive (SRA) with the following sample accession numbers: ERS 3356354- ERS 3356361. The complete study can also be accessed directly using the following URL: http://www.ebi.ac.uk/ena/data/view/ PRJEB32064.
Digital gene expression analysis was carried out using CLC Genomics Workbench v11 to generate BAM (mapping) files, and QSeq Software (DNAStar Inc., Madison, WI, USA) was then used to estimate expression levels. The $\log_2$ (RPKM) values (normalized mapped read values; geometric means of the biological replicate samples) were subsequently used to calculate fold-change values. To identify differentially expressed genes, we used the Student´s t-test (as implemented in Qseq) and corrected for multiple testing using the Benjamini–Hochberg procedure to check the false discovery rate (FDR). In addition to the method implemented in Qseq, we used an alternative method for

normalization and differential gene expression analysis. Mapped reads were log2-transformed and normalized using the quantile method and statistical analysis of the normalized data was carried out using the “empirical analysis of digital gene expression” (EDGE) tool, implemented in CLC Genomics Workbench v8.1. For both methods, a gene was considered significantly differentially expressed with a minimum two-fold change and if the FDR-corrected p-value was less than 0.05.

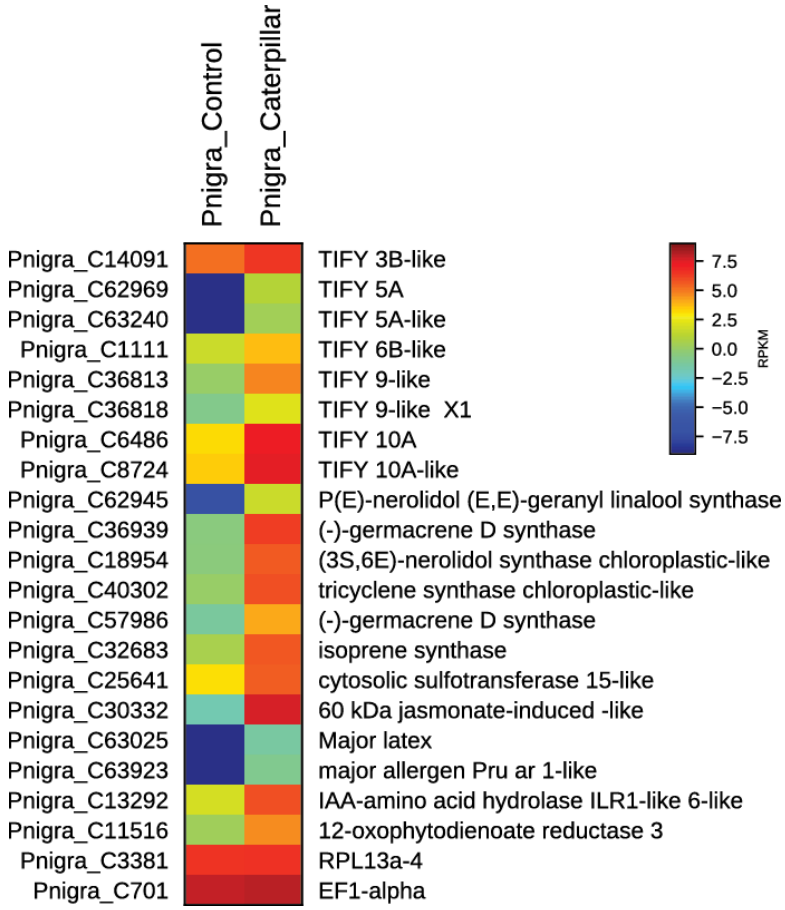

Figure S2: Heat map of 20 exemplary wounding or JA-mediated defense related genes identified in the *P. nigra* transcriptome, for leaves that were damaged by gypsy moth caterpillars (Pnigra_caterpillar) compared to controls (Pnigra_control). The map is based on log2-transformed RPKM values (blue represents low-expressed genes, and red represents highly-expressed genes). RPL13a-4 and EF1-alpha were included as control genes.

Table S2: Proteinase inhibitor levels of black poplar leaves after herbivore damage by gypsy moth caterpillars (caterpillar), previous exposure to HIPVs (HIPV exposed) and a combination of herbivore damaged and previously exposed to HIPVs (HIPV exposed + caterpillar) compared to control plants (control). Different letters indicate significant differences between treatments based on a Kruskal Wallis test with Dunns post hoc (protein content: n.s.; trypsin inhibitor: H = 11.045, p = 0.011; n = 4-6). Shown are means ± SE; n = 4-6.

	control		HIPV exposed		caterpillar		HIPV exposed + caterpillar	
	Mean	± SE	Mean	± SE	Mean	± SE	Mean	± SE
total protein content [mg ml^{-1}]	1.09	0.10	0.81	0.15	0.59	0.09	0.84	0.13
trypsin inhibitor content [µg mg^{-1} protein]	7.71^{a}	0.85	13.88^{ab}	4.36	19.09^{b}	1.87	25.25^{b}	5.73

Table S3: phenylacetaldoxime content in black poplar leaves after herbivore damage by gypsy moth caterpillars (caterpillar), previous exposure to HIPVs (HIPV exposed) and a combination of herbivore damaged and previously exposed to HIPVs (HIPV exposed + caterpillar) compared to control plants (control). Different letters indicate significant differences between treatments based on a Kruskal Wallis test with Dunns post hoc (H = 12.445, p = 0.006; n = 5-6). Shown are means ± SE; n = 5-6.

	control		HIPV exposed		caterpillar		HIPV exposed + caterpillar	
	Mean	±SE	Mean	±SE	Mean	±SE	Mean	±SE
phenylacetaldoxime [µg g^{-1} DW]	0.03^{a}	0.01	0.03^{a}	0.02	0.77^{ab}	0.39	0.68^{b}	0.21

Table S4: Levels of free sugars in black poplar leaves after herbivore damage by gypsy moth caterpillars (caterpillar), previous exposure to HIPVs (HIPV exposed) and a combination of herbivore damaged and previously exposed to HIPVs (HIPV exposed + caterpillar) compared to control plants (control). Different letters indicate significant differences between treatments based on an ANOVA with Tukey´post hoc (sucrose and trisaccharide n.s.; fructose: F = 5.389; p = 0.007) or a Kruskal Wallis test with Dunns post hoc (glucose: H = 10.5; p = 0.033; tetrasaacharide n.s.). Shown are means ± SE; n = 5-6.

	control		HIPV exposed		caterpillar		HIPV exposed+caterpillar	
	Mean	± SE	Mean	± SE	Mean	± SE	Mean	± SE
Glucose	1.27[a]	0.17	1.47[ab]	0.18	3.79[b]	0.66	2.71[ab]	0.80
Fructose	3.71[a]	0.35	3.48[a]	0.38	5.71[b]	0.67	5.45[b]	0.57
Sucrose	29.81	1.57	28.39	1.00	30.11	1.02	30.26	1.03
Trisaccharide	0.29	0.03	0.26	0.02	0.27	0.04	0.28	0.02
Tetrasaccharide	0.04	0.01	0.02	0.00	0.03	0.01	0.03	0.01

10.4 MANUSCRIPT IV – SUPPLEMENTAL DATA

Supplemental material

Title: Ontogenetic differences in black poplar (*Populus nigra*) leaf chemistry influence feeding and oviposition of the poplar leaf beetle *Chrysomela populi*

Authors: Thomas Fabisch[1], Jonathan Gershenzon[1] & Sybille B. Unsicker[1]

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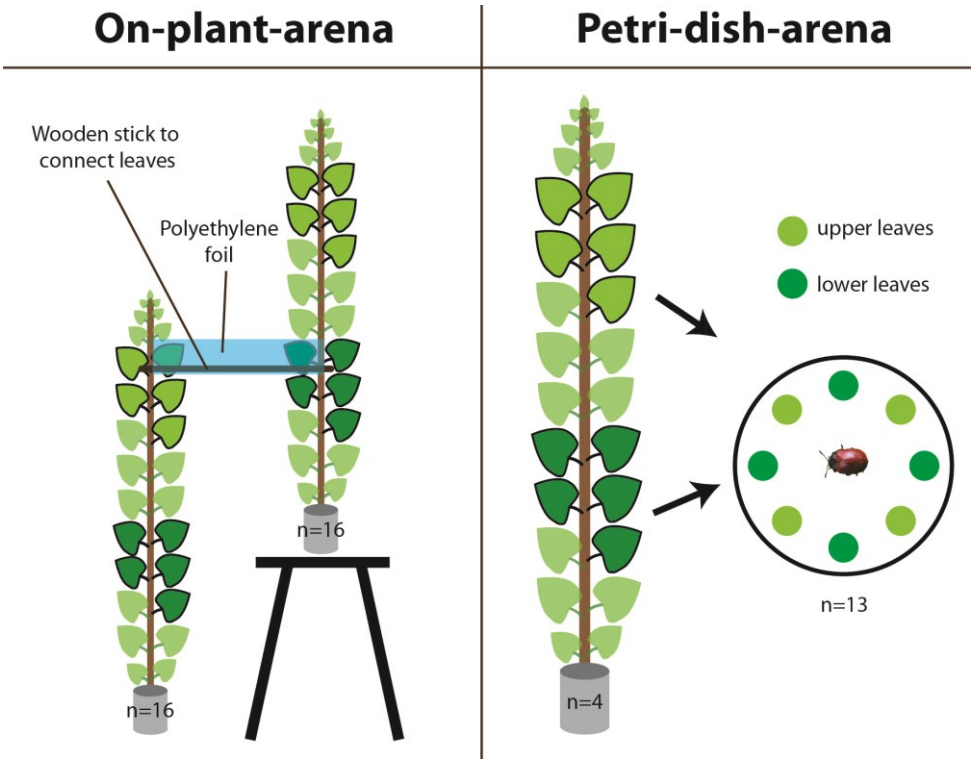


Fig S1: Experimental scheme for the investigation of oviposition- (left) and herbivory (right) preferences of adult *C. populi* leaf beetles on black poplar (*P. nigra*).

To investigate the oviposition behavior, a single beetle pair, consisting of male and female, was enclosed in a tube-like structure with polyethylene bags each containing one of the upper and one of the lower leaves still attached to the trees. To adjust the leaves horizontally one of the two trees of each pair was placed onto a bank. The leaves were connected by a wooden stick to improve the mobility of the beetles between the leaves. The tubes were checked for insect eggs twice a day. After successful oviposition the date as well as the number of eggs was noted. The beetles were enclosed until their second oviposition event. Afterwards they were removed from the plant.

To investigate the amount of *C. populi* damage leaf discs (diameter 16 mm) were cut out of leaves from the upper and the lower leaf pool, respectively, and pinned alternately on petri dishes (n=13). The discs of all four trees used for this experiment were randomly mixed so that each petri dish contained one upper and one lower leaf disc of each plant, resulting in four lower and four upper leaf discs per arena. After the arenas were prepared, one *C. populi* adult beetle was transferred into each arena and was allowed to feed for 24 h. The beetles were randomly taken out of the population to mix males and females. After 24 h the beetles were removed from the arena and the damage on the leaf discs was quantified.

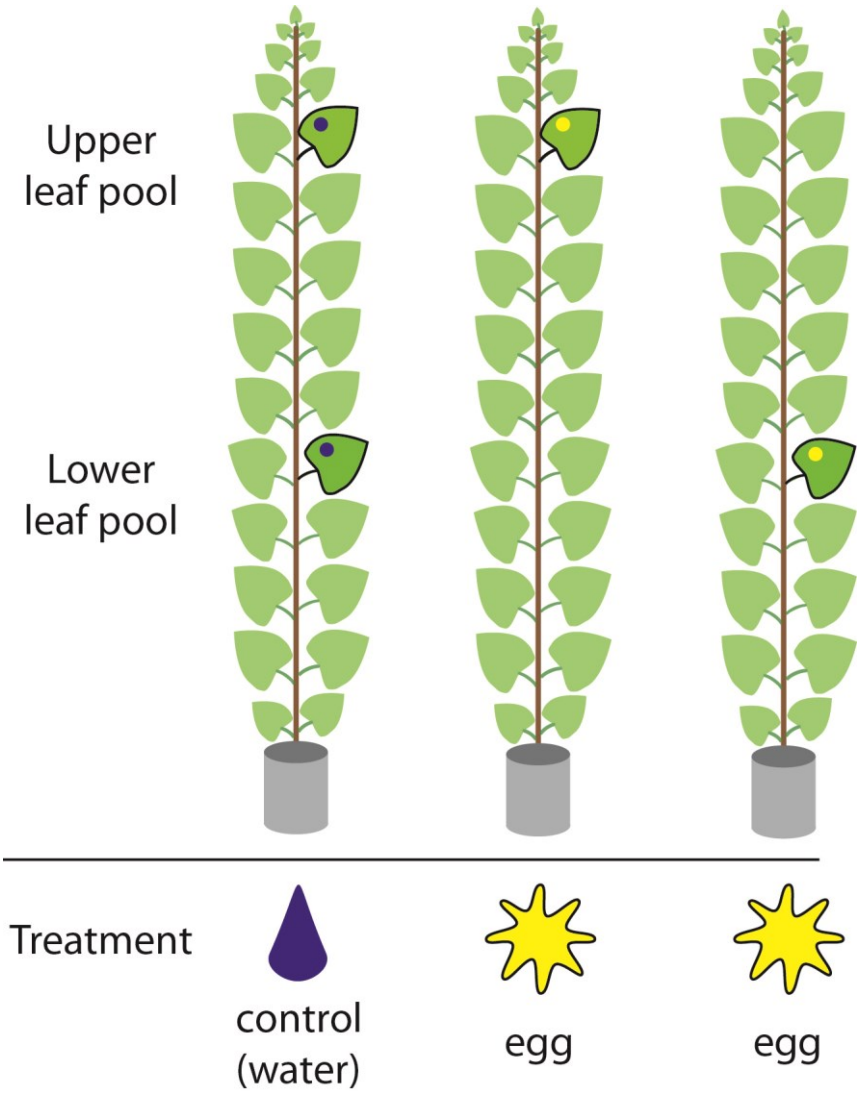
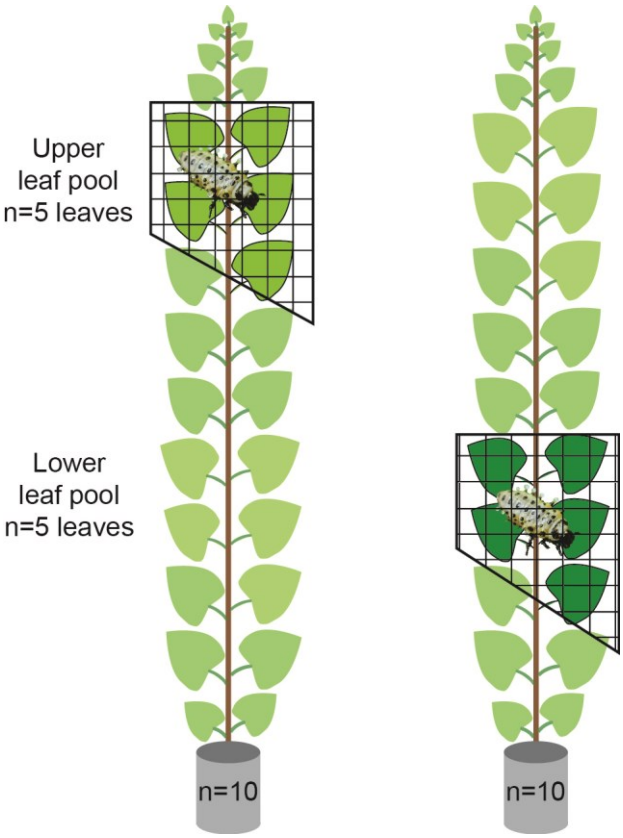


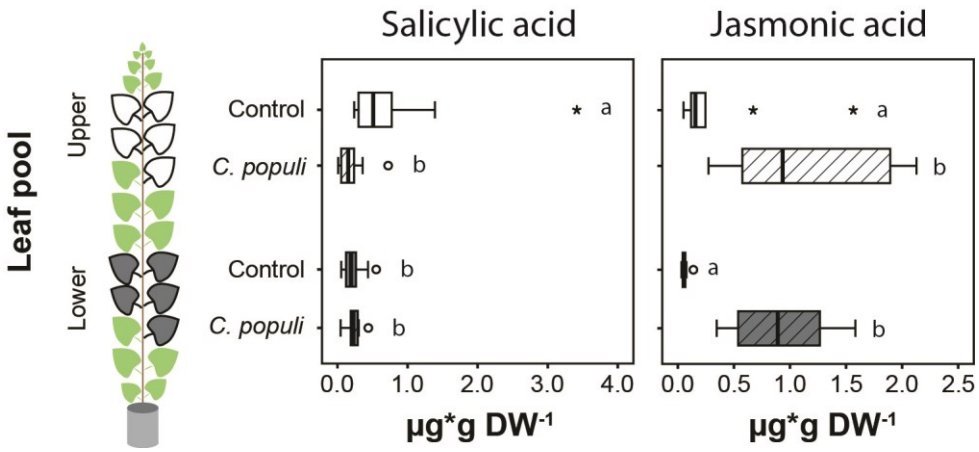
Fig.S2: Experiment scheme for the artificial induction of black poplar defense compounds in leaves belonging to either the upper or the lower leaf pool using *C. populi* egg material

Crushed *C. populi* egg material from eggs harvested one day after oviposition was applied on a pre-defined area (diameter 20 mm) of representative leaves either belonging to the upper leaf pool or the lower leaf pool of young poplar trees (n=10 per treatment). For application a similar amount of egg material than an average clutch weight of *C. populi* was used. The material was applied with a soft brush. Leaves of control trees (n=10, each tree containing two treatments) were treated with water instead. The material was left on the leaves for 3 days before it was gently removed with a soft cloth. Afterwards samples were taken from below the application site using a cork borer (diameter 20 mm). The leaf samples were immediately frozen in liquid nitrogen before they were stored at -20 °C until further use.



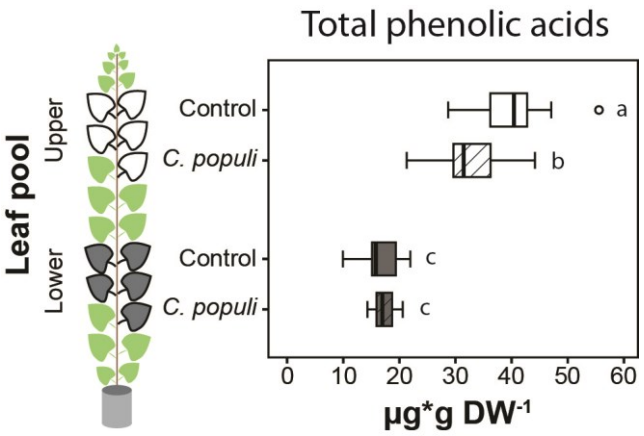
FigS3: Experimental scheme for the larval performance of the chrysomelid leaf beetle *C .populi* on young black poplar (*P. nigra*) trees in dependence of the leaf age

The experiment was performed with 15 freshly hatched larvae per tree, which were transferred from a rearing culture onto single leaves belonging to either younger (upper) or older (lower) leaf pools. The number of replicates was n=10 trees per leaf pool. On each tree used in the experiment only one of the two leaf pools was marked, summing up to 20 trees used in this experiment. The larvae were first caged onto the oldest leaf within the leaf pools until it was almost consumed and subsequently transferred onto the next leaves in apical direction whenever this was necessary. If one pool of larvae had to be transferred, all other larvae were transferred as well. They were allowed to feed on the respective leaf pools for 31 days until all surviving larvae pupated. Every 3 days the mortality and the total weight of the remaining larvae on each leaf pool of each tree were noted to calculate the average larval weight. The performance was repeated in a second experiment also with trees with these leaf pools marked but instead being transferred onto the leaves the larvae originated from eggs oviposited onto this leaves earlier. The number of 15 larvae per pool was realized by reducing the egg clutch to 15 larvae after hatching. During the second experiment the larvae were only allowed to feed for 16 days.



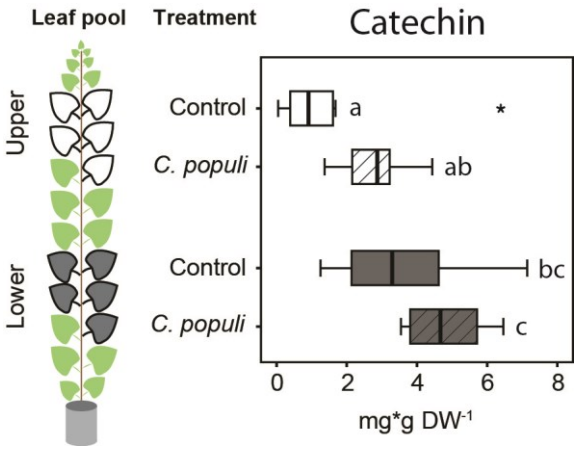
FigS4: Concentrations of stress-related phytohormones in upper and lower leaves of black poplar (*P. nigra*) control trees and trees infested by adults of the chrysomelid leaf beetle *C. populi*

Shown are the concentrations of the two stress-related phytohormones salicylic acid and jasmonic acid in upper and lower black poplar leaves (n=10) after one week of random, non-controlled herbivory and oviposition events by the specialized poplar leaf beetle *C. populi* (dashed boxes) as compared to leaves of non-infested control trees (blank boxes). The control leaves (n=10) were harvested at the start of the experiment to reflect the situation the adult beetles were confronted with when they arrived at the plant. The boxes represent the median ± 1.5 interquartile ranges. Circles represent outliers and asterisks represent extreme outliers. Small letters on the right side of the boxes represent the results of Tukey's post-hoc analysis performed subsequently to a mixed effect model. Results of this model are presented in table 1.



FigS5: Concentrations of total phenolic acids in upper and lower leaf pools of young black poplar (*P. nigra*) control trees and trees infested by the chrysomelid leaf beetle *C. populi*.

Shown are the concentrations of the summed phenolic acids caffeic acid, p-coumaric acid, ferulic acid, cinnamic acid and 3,4-dimethoxycinnamic acid in upper (white bars) and lower (grey bars) black poplar leaves (n=10) after one week of random, non-controlled herbivory and oviposition events by the specialized poplar leaf beetle *C. populi* (dashed boxes) as compared to leaves of non-infested control trees (blank boxes). The control leaves (n=10) were harvested at the start of the experiment to reflect the situation the adult beetles were confronted with when they arrived at the plant. The boxes represent the median ± 1.5 interquartile ranges. Circles represent outliers. Small letters on the right side of the boxes represent the results of Tukey's post-hoc analysis performed subsequently to a mixed effect model. Results of this model are presented in table 1.



FigS6: Concentrations of the flavonoid catechin in upper and lower leaf pools of young black poplar (*P. nigra*) control trees and trees infested by the chrysomelid leaf beetle *C. populi*.

Shown are the concentrations of the catechin in upper (white bars) and lower (grey bars) black poplar leaves (n=10) after one week of random, non-controlled herbivory and oviposition events by the specialized poplar leaf beetle *C. populi* (dashed boxes) as compared to leaves of non-infested control trees (blank boxes). The control leaves (n=10) were harvested at the start of the experiment to reflect the situation the adult beetles were confronted with when they arrived at the plant. The boxes represent the median ± 1.5 interquartile ranges. Asterisks represent extreme outliers. Small letters on the right side of the boxes represent the results of Tukey's post-hoc analysis performed subsequently to a mixed effect model. Results of this model are presented in table 1.

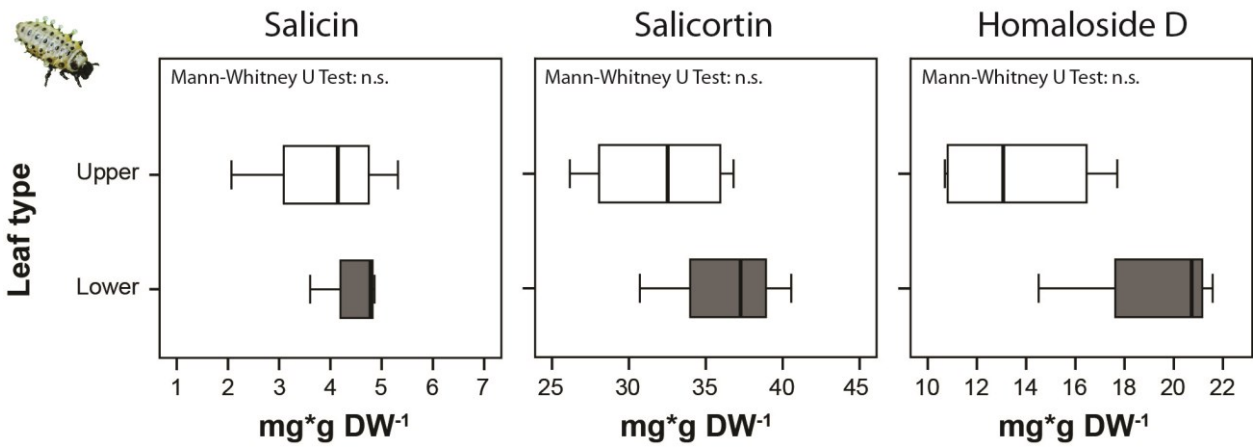


Fig S7: Salicinoid levels in upper and lower leaves of young *P. nigra* trees after 8 days of herbivory by *C. populi* larvae.

The data derived from leaf samples collected during the larval performance experiment. The leaf samples (n=3 from lower leaves and n=4 from upper leaves) derived from the leaves exposed to prior egg deposition and feeding events of the hatched larvae. The samples were taken when the leaf was almost consumed by the young larvae at a time point (8 days post hatching) when they were transferred onto the next leaf. The boxes represent the median ± 1.5 interquartile ranges. The statistical tests are depicted inside the boxes.

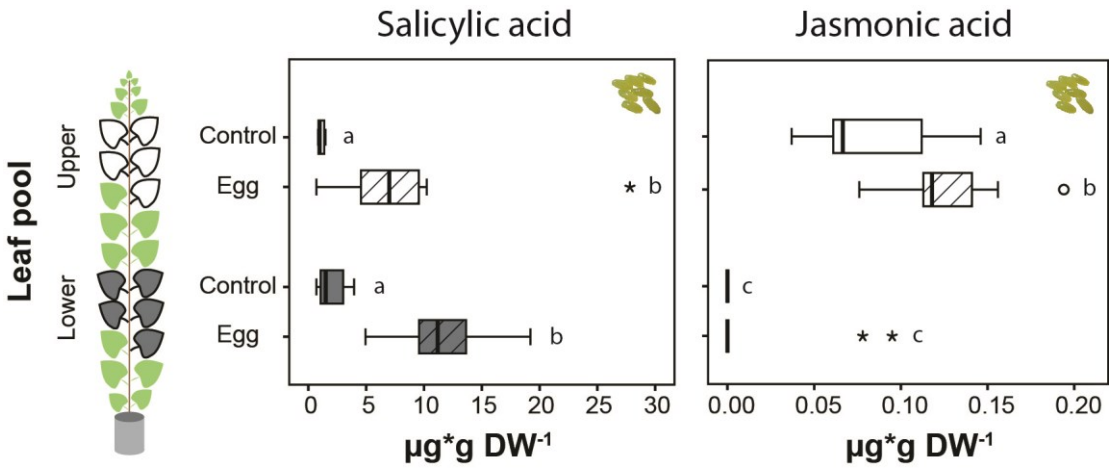
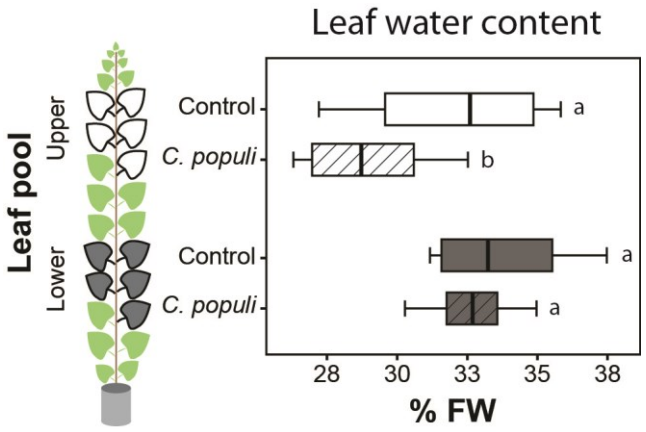


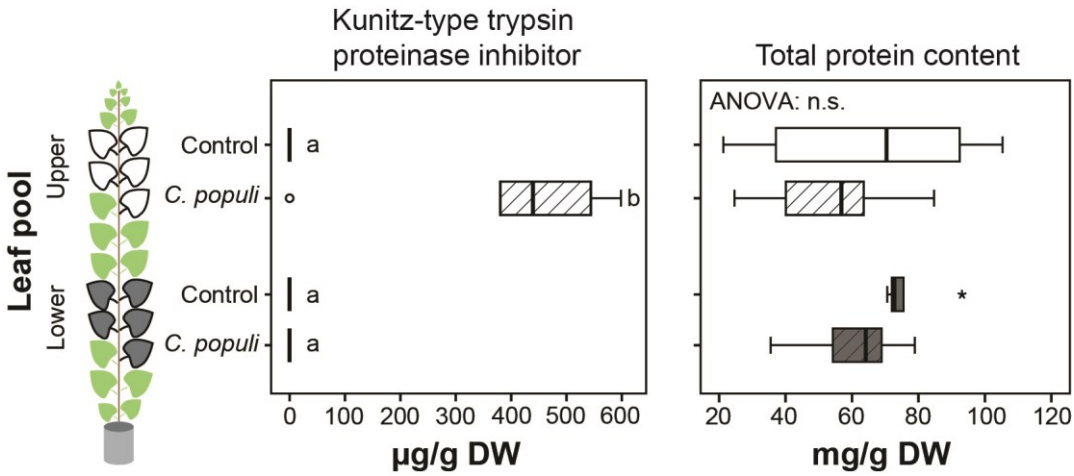
Fig S8: Egg-induced concentrations of stress-related phytohormones in black poplar (*P. nigra*).

Depicted are the levels of the phytohormones jasmonic acid and salicylic acid directly beneath the treatment site (diameter 20 mm) in upper (white boxes-dashed area, control n=6, egg n=9) and lower (grey boxes-dashed area, control n=8, egg n=9) leaves of black poplar trees 3 days after simulation of oviposition by *C. populi* as compared to water-treated control leaves (non-dashed white/grey boxes). The odd number of replicates resulted from the loss of some samples due to contamination during the processing. The simulation of oviposition was artificially performed with crushed eggs using a similar amount of egg material than an average clutch weight of *C. populi*. Circles represent outliers and asterisks represent extreme outliers. Small letters on the right side of the boxes represent the results of Tukey's post-hoc analysis performed subsequently to a mixed effect model. Results of this model are presented in table 1.



FigS9: Water content in upper and lower leaf pools of young black poplar (*P. nigra*) control trees and trees infested by the chrysomelid leaf beetle *C. populi*.

Shown is the water content in upper (white bars) and lower (grey bars) black poplar leaves (n=10) after one week of random, non-controlled herbivory and oviposition events by the specialized poplar leaf beetle *C. populi* (dashed boxes) as compared to leaves of non-infested control trees (blank boxes). The control leaves (n=10) were harvested at the start of the experiment to reflect the situation the adult beetles were confronted with when they arrived at the plant. The boxes represent the median ± 1.5 interquartile ranges. Small letters on the right side of the boxes represent the results of Tukey's post-hoc analysis performed subsequently to a mixed effect model. Results of this model are presented in table 1.



FigS10: Kuntiz-type trypsin proteinase inhibitor activity in upper and lower leaf pools of young black poplar (*P. nigra*) control trees and trees infested by the chrysomelid leaf beetle *C. populi*.

Shown is the KTI activity in upper (white bars) and lower (grey bars) black poplar leaves (n=6) after one week of random, non-controlled herbivory and oviposition events by the specialized poplar leaf beetle *C. populi* (dashed boxes) as compared to leaves of non-infested control trees (blank boxes). The control leaves (n=6) were harvested at the start of the experiment to reflect the situation the adult beetles were confronted with when they arrived at the plant. The boxes represent the median ± 1.5 interquartile ranges. Small letters on the right side of the boxes represent the results of Tukey's post-hoc analysis performed subsequently to a mixed effect model.

Table S1 MRM settings used for the identification and quantification of amino acids via LC/MS/MS

Amino acid	Retention time (min)	Declustering potential (V)	Entrance potential (V)	Collision energy (eV)	Mass of parent ion (da)	Mass of daughter ion (da)
Ala	0.4	20	5.5	17	90.1	44.1
Ser	0.4	20	4.5	15	106.0	60.1
Pro	0.6	20	7.5	19	116.1	70.0
Val	0.6	20	5.0	13	118.1	72.2
Thr	0.4	20	4.5	13	120.1	74.2
Ile+Leu	0.9	20	4.5	13	132.2	86.1
Asp	0.4	20	5.5	19	134.1	74.1
Glu	0.4	20	5.5	15	148.1	102.1
His	0.4	20	5.5	17	156.2	110.1
Phe	2.1	20	6.0	17	166.2	120.2
Tyr	1.1	20	7.0	17	182.1	136.2
Asn	0.4	20	4.5	21	133.1	74.1
Gln	0.4	20	6.0	13	147.1	130.0
Trp	2.8	20	4.5	13	205.2	188.1
Lys	0.4	20	6.0	23	147.1	84.1
labAla	0.4	20	5.5	17	94.1	47.1
labSer	0.4	20	4.5	15	110.0	63.1
labPro	0.6	20	7.5	19	122.1	75.0
labVal	0.6	20	4.5	15	124.1	77.2
labThr	0.4	20	5.0	13	125.1	78.2
labIle	0.9	20	4.5	13	139.2	92.1
labAsp	0.4	20	10.0	19	139.1	77.1
labGlu	0.4	20	5.5	15	154.1	107.1
labHis	0.4	20	5.5	17	165.2	118.1
labPhe	2.1	20	6.0	17	176.2	129.2
labTyr	1.1	20	7.0	17	192.1	145.2
labGln	0.4	20	6.0	13	154.1	136.0
labTrp	2.8	20	4.5	13	218.2	200.1
labLys	0.4	20	6.0	23	155.1	90.1

10.5 ERGÄNZENDE ÜBERSICHT ZUM EIGENANTEIL DER MANUSKRIPTE

Manuskript Nr. 1

Kurzreferenz: Fabisch et al. 2019

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Unterschrift Kandidat/-in Unterschrift Betreuer/-in (Mitglied der Fakultät)

Manuskript Nr. 3

Kurzreferenz: Lackner et al. unpublished

Beitrag des Doktoranden / der Doktorandin

Beitrag des Doktoranden / der Doktorandin zu Abbildungen, die experimentelle Daten wiedergeben (nur für Originalartikel):

Abbildung(en) # 1-4	☐	100% (die in dieser Abbildung wiedergegebenen Daten entstammen vollständig experimentellen Arbeiten, die der Kandidat/die Kandidatin durchgeführt hat)
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Manuskript Nr. 4

Kurzreferenz: Fabisch et al. unpublished

Beitrag des Doktoranden / der Doktorandin

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