Phenolics in black poplar (*Populus nigra*) - Patterns of abundance and processing in herbivores

Dissertation

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von

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1. Introduction

1.1. General aspects of plant defense against herbivores

The primordial origins of the mutual relationship between plants and their herbivores date back approximately 400 million years (Labandeira 2007) and represent the beginning of one of the oldest interactions between organisms in the history of life. During their long-lasting co-existence, both plants and herbivores have experienced cycles of adaptation and counter-adaptation that drove their evolution. Plants obviously developed under the pressure to avoid herbivory, while herbivores had to maintain a food source. The result is a variety of defense mechanisms against herbivores on the plant side and manifold counter-adaptations by the herbivores with the aim to render the defenses less effective.

In general, two types of plant defenses are distinguished: direct and indirect defense. Direct defenses are immediately adverse to the attacker and theoretically harm all types of animals. In contrast, indirect defenses involve a third trophic level, i.e. natural enemies of the herbivores, and affect mostly arthropods. The most frequently observed and best studied way for plants to mediate both types of defenses is by the deployment of chemicals. These chemicals belong to a class of compounds called “secondary metabolites”; a term applied to all plant substances that were historically considered non-essential (Wink 2003), although this classical notion has been subjected to increasing criticism (Neilson *et al.* 2013). Secondary metabolites involved in direct defense are deterrent or toxic to herbivores. Their counterparts, the indirect defense secondary metabolites, are typically volatile molecules that advertise the presence of herbivores to their natural enemies. In accordance with the scope of this dissertation, the following paragraphs provide a general introduction to direct defense employing secondary metabolites. Additionally, the direct defenses of the genus *Populus* in the family of the Salicaceae are summarized. Throughout the document, the term direct defense is used in the context of non-volatile secondary defense metabolites of terrestrial plants, unless further specified. Likewise, the term herbivore is used as a synonym for leaf-chewing insects if other animals like vertebrates are not explicitly included.

1.2. Direct plant defense

A short history of direct defense research

In the course of the last centuries, phytochemists have identified a bewildering quantity of secondary metabolites. Accurate estimates of the actual number are unavailable. However, around the turn of the millennium an estimate was made of approximately 200,000 compounds (Dixon & Strack 2003). A primary intention of identifying secondary metabolites was the search for novel drugs (Balunas & Kinghorn 2005). Today, the importance of botanical drugs is diminishing (Li & Vederas 2009), but most of the new discoveries are still related to a potential pharmacological application. The utilization of plants in human medicines has several thousand years of tradition, but it took mankind until the late 19th century to realize the bioactivity of some secondary metabolites against other organisms. Early work carried out by Stahl (1888) lead to the insight that plant "excretions" must contain substances that confer protection against some herbivores but not against others. After decades of relatively few progresses, Fraenkel (1959) came up with the revolutionary hypothesis that the raison d'être of plant secondary metabolites is solely to combat herbivores. Although this radical theory is no longer tenable from today's point of view, it constituted the beginning of an ongoing research era that addresses phytochemical-mediated interactions of plants with their environment. The accumulating knowledge of these scientific efforts has led to the notion that many secondary plant compounds are produced to interact with the biotic or abiotic environment (Hartmann 2007). Among these compounds, some have emerged to act as direct defenses against herbivores. Prominent examples of such compounds were identified because of their inherent efficacy (e.g. pyrethrins) or their presence in crops and widely used model plants (e.g. glucosinolates). However, due to the increased attention that secondary metabolites have received since the seminal paper of Fraenkel (1959), our knowledge is not merely restricted to the efficacy of direct defense compounds, but includes insights about occurrence, biosynthesis and mode of action. For example it is known that only few direct defense compounds are immediately noxious to herbivores in their natural form. Such compounds have discrete targets which typically occur only in herbivores and not in plants. Pyrethrins, for example, interfere with specific ion channels located in the nervous system of insects and higher animals (Wolansky & Harrill 2008). In contrast, many other direct defense compounds are only activated in the case of tissue damage and are otherwise stored in an inactive form. In toxicology, such inactive forms, which are eventually converted into a toxin, are called protoxins, and the reaction cascade ultimately leading to toxicity is termed bioactivation. The bioactivation of protoxic direct defense compounds is often carried out by separately stored

enzymes or enzymes endogenous to the herbivore creating a binary defense system (prototoxin and activating enzyme) that minimizes auto-toxicity. If the bioactivating enzymes are plant derived, they are typically localized in other compartments than the protoxin and both components only come into contact when the tissue disintegrates, for example in the case of damage to chewing herbivores.

Bioactivation of direct defense compounds

A wide-spread pattern in the plant kingdom is to store defensive compounds as protoxic glycosides, usually as β-glucosides (many authors still prefer the general term "glycoside"), whose bioactivation is initiated by glucosidase-catalyzed deglycosylation (**Fig. 1**). Classical examples of direct defense compounds activated by glucosidases are the **cyanogenic glycosides** (reviewed by Zagrobelny *et al.* 2004), which occur in more than 2500 plant species. Intact cyanogenic glycosides are stored in the vacuole and can be cleaved by plant glucosidases and possibly also by glucosidases of bacteria, fungi and animals. The aglycone formed upon glucosidase action spontaneously decomposes to form toxic HCN at pH values higher than 6 and in more acidic environments the decomposition of the aglycone may be catalyzed by a second type of bioactivating enzyme: α-hydroxynitrile lyases. HCN is a very potent toxin and interferes with cytochrome c oxidases of the respiratory pathway in a complex manner (Leavesley *et al.* 2008). Compared to cyanogenic glycosides, many other direct defenses do not target single enzymes, but produce reactive species with presumably low target specificity. **Glucosinolates** occur in many plants of the order Brassicales and their characteristic element is a *β*-thioglucosidic sulfonated oxime connected to a variable portion, whose nature classifies the glucosinolate as aliphatic, benzenic or indolic. The enzymes responsible for glucosinolate bioactivation are specific thioglucosidases, the so-called myrosinases, which are exclusively localized in myrosin and guard cells well separated from their substrates *in planta* (Halkier & Gershenzon 2006). When the compartmentation disintegrates, the myrosinase catalyzes the cleavage of the S-glycosidic bond and the released aglycone decomposes to form isothiocyanates, nitriles and other molecules (Halkier & Gershenzon 2006; Pentzold *et al.* 2013). These compounds are considered toxic due to their electrophilic nature and it is assumed that they alkylate proteins or nucleotides. Other glycosidic defense compounds, such as **benzoxazinoids** and **iridoid glycosides**, also form electrophiles after deglucosylation (Dobler, Petschenka & Pankoke 2011). In fact, the reactivity of the imidoquinines formed upon benzoxaninoid decomposition is relatively well understood since these metabolites were shown to add to the SH groups of cysteine containing proteins (Dixon *et al.* 2012). However, the

reactivity of direct defense compounds may also result from the bioactivation of non-glycosides (**Fig. 1**), such as the **pyrrolizidine alkaloids**. These protoxins are stored as non-toxic N-oxides (structure not shown), but are readily reduced to tertiary amines, which are oxidized to reactive electrophilic pyrrolic intermediates by P450 mono-oxygenases in the herbivores (Hartmann 1999).

These protoxins usually occur in herbaceous plants, while woody plants instead produce **phenolics** that are presumed to have protoxic properties. The common feature of these compounds is an aromatic ring with at least one OH group, although more OH substitutions occur frequently, and therefore phenolics include a large range of compounds with only low structural similarity. Small phenolics are often glycosides, but so far there is no evidence that deglycosylation plays a role in their bioactivation. Originally it was believed that phenolics exhibit anti-nutritive effects instead of being protoxic. One of the first types of phenolics to which direct defense properties were attributed were the condensed tannins occurring in many tree species (Feeny 1970). The astringency of condensed tannins, i.e. their ability to precipitate protein, was supposed to confer universal anti-nutritive properties. However, a lack of a broad spectrum activity against herbivores in some studies (Ayres *et al.* 1997) but not in others (Forkner, Marquis & Lill 2004) has led to an ongoing debate about role of condensed tannins in plant-insect interactions. The bioactivity of smaller phenolics, which are less likely to precipitate protein, has given rise to a more recent theory about their mode of action: the suggestion that diphenolic compounds are bioactivated *in vivo* to form reactive quinones (**Fig. 1**, Appel 1993). This oxidation can take place spontaneously under appropriate redox conditions, but is also catalyzed by enzymes, such as polyphenol oxidases or peroxidases (Duffey & Stout 1996; Pourcel *et al.* 2007). Along with the formation of noxious quinones, the enzymatic reaction can produce reactive oxygen species as byproducts, such as the superoxide anion radical (Appel 1993). The physiological consequences of quinone and reactive oxygen species formation is commonly described as “oxidative stress” (Barbehenn, Poopat & Spencer 2003) and the intermediates have been proposed to react with the amino or thiol residues of proteins (Duffey & Stout 1996) similar to the active metabolites of other direct defense compounds. However, the last decades have provided little progress in proving this mode of action. Our lack of understanding is well indicated by the inconsistencies of the reaction pathways and products proposed by different authors (e.g. Felton *et al.* 1992; Appel 1993; Haruta, Pedersen & Constabel 2001). It should be noted that phenolics are not generally protoxic and some representatives, such as flavonoids, are considered to have antioxidant properties that confer the ability to quench reactive oxygen species (Hernandez *et al.* 2009).

Understanding this Janus character of phenolics is a challenge requiring much more effort to investigate the metabolic role of dietary phenolics in herbivores.

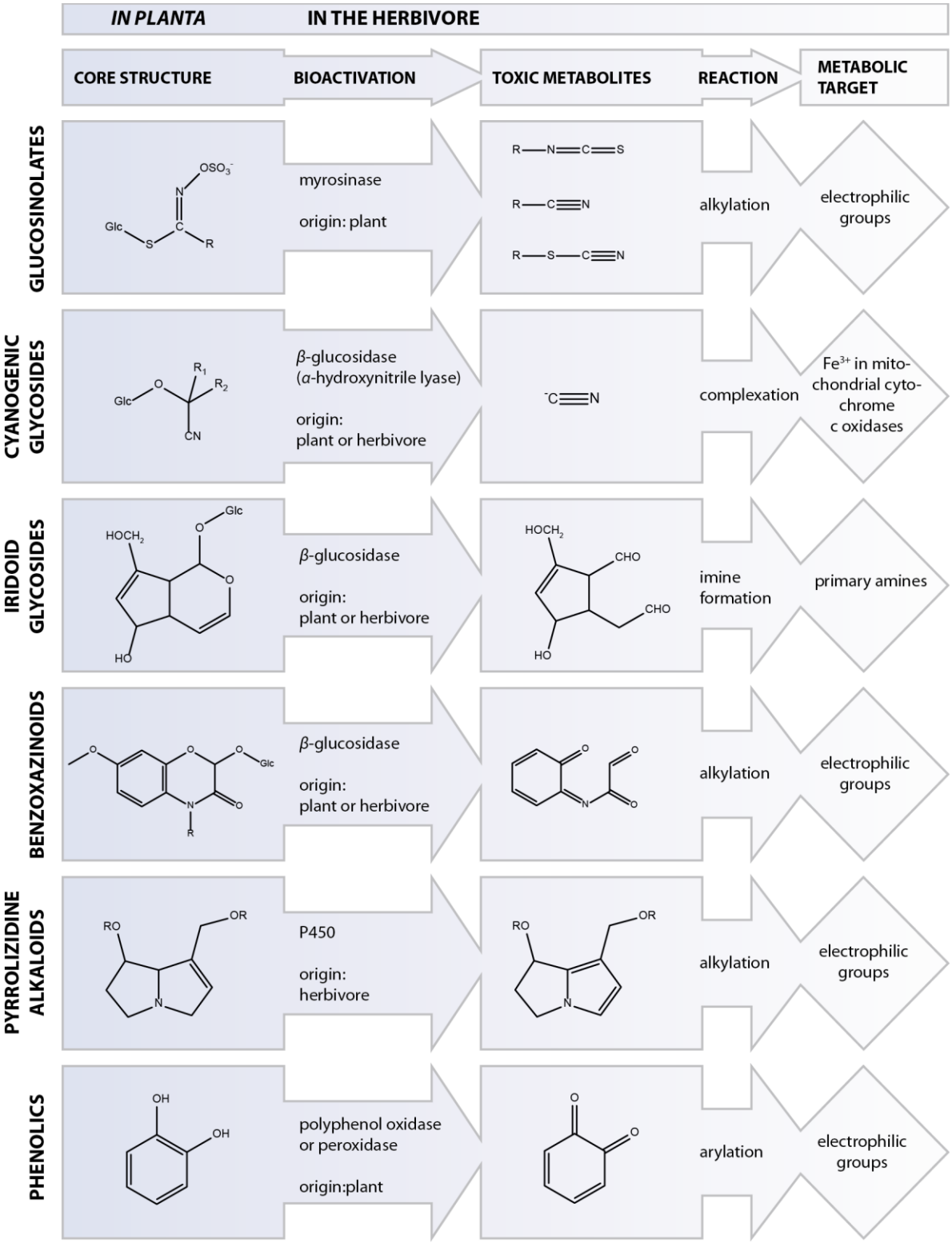

Fig. 1. Core structure, bioactivation enzymes and toxic derivatives of various direct defense compounds that are stored in the plant as protoxins, along with their suggested metabolic targets. Possible intermediates in bioactivation are not shown.

1.3. Direct defense compounds from the plant perspective

The obvious evolutionary success of herbivorous animals illustrates that the defense measures deployed by plants do not provide total protection. In fact, the evolutionary relationship between plants and herbivores is usually described as an arms race (Rasmann 2014): plants continually develop new defenses that are sooner or later overcome by the adaptive abilities of herbivores. These cycles of adaptation and counter-adaptation are believed to be a major factor in driving the species diversification of both plants and herbivores and might be better termed a dynamic equilibrium than an arms race. The evolutionary histories of plants and their herbivores are not verifiable with current scientific methods, but certain questions can be tested, such as if defenses confer a detectable fitness advantage on plants.

The production of direct defense compounds requires resources that can no longer be allocated to growth and reproduction. Besides the fixed carbon, nitrogen and other elements that are incorporated into the compounds themselves (and the cofactors required for bioactivation), other costs include the resources for their biosynthesis, transport and storage. Moreover plant defenses can have indirect, ecological costs that originate from their negative impact on mutualistic animals, such as pollinators, or a retardation of growth compared to the competing vegetation (Strauss *et al.* 2002). In temperate zones, where a deciduous lifestyle is widespread, the fluctuations of photosynthate availability and herbivore occurrence make the timing of defense also important. A common strategy of plants to cope with the dynamic nature of herbivory is the induction of defenses: In contrast to constitutive defenses, which are permanently present, inducible defenses are only activated or increased when leaf damage is detected (Zangerl 2003). Induced defense is often triggered by phytohormones that are biosynthesized from membrane lipids of damaged cells (Erb, Meldau & Howe 2012) and can occur at the site of damage (locally) and in adjacent, undamaged plant organs (systemic). Compared to constitutive defenses, induced defenses take extra time to be deployed, but help the plant to save resources, among other advantages (Zangerl 2003). Thus, a large variety of factors influences the cost of defense.

In an attempt to predict the abundance of plant defenses researchers have developed various hypotheses, such as the optimal defense theory (McKey 1974), the carbon-nutrient balance hypothesis (Bryant, Chapin & Klein 1983) and the growth-differentiation balance hypothesis (Herms & Mattson 1992). Most of these theories have been shown to lack general validity (e.g. (Berenbaum 1995; Hamilton *et al.* 2001), illustrating that our understanding of the costs of plant defenses is still limited. A meta-study conducted by Strauss *et al.* (2002) analyzing 33

articles came to the conclusion that 76 % of the studies detected a cost of defense; however, the magnitude of this cost was very variable.

Similar to detection of the costs, measuring the benefits of defense is a Sisyphean task. Each defense compound can have an ecosystem-wide impact by influencing the whole community of animals associated with a plant. Such animal communities contain multiple trophic levels and include species that are antagonistic and beneficial for the plant, both of which can be influenced negatively or positively (Ibanez, Gallet & Despres 2012). Additionally, the multifunctional role of some defense compounds may benefit or harm the producer in different ways than protection against herbivores. The complexity of possible feedbacks caused by every single defense compound, which can be variable with time and space, indicates that determining the true benefit of each substance is experimentally unfeasible even within short timeframes. Therefore, experimental approaches are often simplified to just correlate the abundance of direct defenses with herbivore damage, plant size or reproductive success. The result of such approaches indicates that such correlations are strongly dependent on the composition of the herbivore community. For example, Bidart-Bouzat and Kliebenstein (2008) found in a common garden experiment that the glucosinolate levels of *A. thaliana* were positively correlated with herbivore damage and negatively correlated with plant fitness (i.e. seed production). As this result was contrary to expectations, it was concluded that the high density of specialized herbivores (see below) was responsible. Herbivory by specialists also played a role in an experiment investigating the role of pyrrolizidine alkaloids in *Senecio jacobea* (Macel & Klinkhamer 2010). The study was conducted at two different sites, one with and one without naturally occurring specialist. At the site with specialists, herbivory was positively correlated with the pyrrolozidine alkaloid concentration, while no significant correlation was found at the site with mainly generalists. Another field study with *Brassica oleracea* found no connection between the glucosinolate content and leaf damage at all (Moyes *et al.* 2000). Convincing evidence for the anti-herbivore efficacy of induced plant defense was shown by Kessler, Halitschke and Baldwin (2004): when genes important for the biosynthesis of wound-signaling phytohormones were silenced in *Nicotiana attenuata*, the plants were far more vulnerable to generalist and specialist herbivores. Although this crude intervention in plant metabolism allows only general insights in the importance of induced defense, more subtle transgenic approaches silencing only one specific defense trait may allow conclusions on a finer scale. Apart from the legal situation concerning the release of transgenic plants in nature, such experiments are restricted by the interdependence of plant metabolic pathways, which make a targeted manipulation of a single defense compound almost impossible.

1.4. Direct defense compounds from the herbivore perspective

Our current knowledge suggests that herbivores are unlikely to encounter an undefended host plant in nature. Although selective feeding on plant tissues with low levels of defense can minimize the uptake of direct defense compounds (Pentzold *et al.* 2013), total evasion is unrealistic. Deriving from this assumption, it is commonly believed that herbivores are compelled to adapt to direct defenses in order to secure access to food. However, the enormous evolutionary diversification of plant direct defenses make universal adaptation to all harmful secondary metabolites impossible and therefore various degrees of specialization have occurred on the herbivore side. Very specialized herbivores can be immune to some direct defense compounds and may even exploit them for their own purposes, but are restricted to a few plant species (see below). In turn, a low degree of specialization permits only a moderate adaption to a larger diversity of defense compounds, but increases host range (Li *et al.* 2004). This trade-off has led to the rough discrimination of herbivores into two categories, specialists and generalists, or three categories, monophagous, oligophagous and polyphagous herbivores, (*sensu* Schoonhoven, van Loon & Dicke 2005). The generally observed correlation between an increasing degree of specialization and the ability to cope with direct defense compounds is believed to be driven by metabolic adaptations of herbivores (Després, David & Gallet 2007). In this respect three mechanisms are of major importance: detoxification, avoidance and sequestration (note: behavioral adaptions are not considered).

Detoxification is the omnipresent capability of organisms to transform xenobiotics, i.e. foreign chemical substances, into harmless metabolites and ultimately store or excrete them. Depending on the nature of the toxin, the process of detoxification involves up to three steps: conversion into a metabolizable form (phase I), conjugation (phase II) and storage or excretion (phase III). These processes are comparatively well understood in humans, but our knowledge about detoxification in herbivores is limited. Certain mechanisms of detoxification appear to be widespread across living organisms. For example, phase I transformations of xenobiotics often include oxidation by cytochrome P450 monooxygenases or reduction by the ascorbate or tocopherol redox systems, depending on their oxidative status. P450-catalyzed oxidation is an important step in the detoxification of pyrrolizidine alkaloids and artificial insecticides; however, the reaction products have not yet been identified (Li *et al.* 2004; Després, David & Gallet 2007). Phase I reductions may be carried out by ascorbate and tocopherol, which are higher in herbivores species feeding on phenol-rich plants (Barbehenn *et al.* 2001; Barbehenn, Walker & Uddin 2003), but there is no direct proof that this observation is related to the reaction with prooxidant metabolites of phenolics, such as quinones or reactive oxygen species

(see above). Even when xenobiotics are transformed into phase I metabolites, a direct elimination is often not possible due to low water solubility, high reactivity or possible reactivation. In such cases a conjugation, i.e. the attachment of a sugar derivative, amino acid or small peptide, is necessary (phase II). Frequently observed conjugations in insects are catalyzed by enzymes of the glutathione-transferase (GST) and UDP-glycosyltransferase (UGT) superfamilies and are well-known to participate in the detoxification of direct defense compounds (Després, David & Gallet 2007). Conjugated metabolites are excretable and can be eliminated in the feces or by incorporation into the exuvia. In general, detoxification is often limited by the activity of the enzymes involved or by the amount of substrates available. Some detoxification steps can be carried out by endosymbionts (Hansen & Moran 2014) so that the cost for the herbivore host may be lower. However, the whole process is thought to be metabolically costly.

In contrast to detoxification, **avoidance** strategies intervene earlier in the interaction of plants and their herbivores and prevent the bioactivation of protoxic defense compounds to toxins. Alternatively, the metabolic target of a toxin can be altered in a way that it is no more susceptible. Several general mechanisms of avoidance are known. For example, it has been hypothesized that the alkaline gut fluid of many lepidopteran herbivores obstructs the activity of plant derived glucosidases and suppresses the bioactivation of glycosidic defense compounds (Fitzgerald 2008). In addition, herbivores can decrease the expression of their own digestive glucosidases when exposed to glycosidic protoxins, leading to a higher tolerance against such defense compounds (Lindroth 1988b; Desroches *et al.* 1997; Ballhorn, Kautz & Lieberei 2010). More specialized insects can avoid toxicity by converting protoxins into harmless metabolites or compounds that are unsuitable substrates for bioactivating enzymes (Engler, Spencer & Gilbert 2000; Ratzka *et al.* 2002; Falk & Gershenzon 2007).

Highly specialized herbivores capable of an effective detoxification or avoidance may develop the ability to s**equester** direct defense compound produced by their host plant. Sequestration is the ability of herbivores to accumulate native or modified direct defense compounds in the body in order to use them against their own enemies. Although the uptake, transport and deposition of plant defenses requires sophisticated metabolic and storage capabilities, sequestration is widespread among herbivores. The processes involved are often compound-specific and therefore sequestering species are limited to certain secondary metabolites produced by a narrow range of host plants. The number of sequestration strategies reflects the diversity of secondary metabolites and will not be addressed in detail here (see Opitz & Müller 2009 for more detailed information). However, it is a common pattern that the direct defense

compounds suitable for sequestration stimulate feeding of the corresponding insects, so that the plant`s protective strategy backfires and herbivory can be eventually higher than without any defense compounds (see above). Some herbivore species even rely on the sequestered compounds as precursors for sex pheromones (Opitz & Müller 2009). Such examples illustrate that the close association to specific secondary metabolites is also restrictive for the sequestering herbivores, as host plant switches are not favored.

Herbivores unable to quantitatively cope with dietary defense compounds (using any of the three mechanisms mentioned above), will suffer physiological disadvantages. Researchers often use performance indicators, such as developmental times, weight gain, pupal weights and mortality as measure for the severity of the intoxication and correlate them to the abundance of a defense compound in order to prove its efficacy. Comparisons of herbivores fed artificial diet or plant material supplemented with the putative compound and control groups are typical experimental strategies (e.g. Ayres *et al.* 1997). More comprehensive approaches test artificial diets with different concentrations or make use of the natural variation of defense compounds offering plant genotypes that vary in the levels of the compound of interest (e.g. Hemming & Lindroth 1995). However, artificial diets often lack possible cofactors necessary for bioactivation, while plant genotypes differ in other aspects than the desired trait. More recently transgenic plants have offered the opportunity to minimize this undesired variation (e.g. Jassbi *et al.* 2008). Nevertheless, there is evidence that the close inter-connection of plant metabolic pathways does not often permit selective manipulation of only one secondary metabolite.

1.5. Direct defense compounds in the model system *Populus*

In the temperate zones, the genera *Populus* and *Salix* are the major representatives of the family of the Salicaceae. Poplars (*Populus*) possess a variety of direct defenses against herbivores, including volatile organic compounds, non-volatile secondary metabolites and defensive proteins (Philippe & Bohlmann 2007). According to our current knowledge, poplars rely on non-volatile phenolic secondary metabolites as direct defenses, like many other trees of this climatic zone (e.g. *Quercus* or *Betula* species). In fact, poplars produce large amount of two classes of phenolics: condensed tannins and the salicinoids (also termed phenolic glycosides or salicylates). Condensed tannins occur in many plant species, and high amounts are typical for boreal deciduous trees, such as poplar, where concentrations of more than 20 % of the foliage dry weight have been reported (Donaldson *et al.* 2006). Although condensed tannins were formerly believed to be broad-spectrum direct defense compounds (see above), there is currently little evidence that these compounds play a significant role against insect herbivores in poplar (Hemming & Lindroth 1995; Barbehenn *et al.* 2009). In contrast, the

salicinoids are a key factor in the insect-plant interactions of poplars. Salicinoids significantly affect the behavior and fitness of vertebrate and invertebrate generalist herbivores (Lindroth 1991; Ruuhola, Tikkanen & Tahvanainen 2001; Diner *et al.* 2009), but are also known to be sequestered by specialist leaf beetles (Burse *et al.* 2009). To date approximately 20-30 different salicinoids (manuscript II) have been described and occur in a concentration range similar to the condensed tannins. Due to their activity and abundance, salicinoids are likely to shape the chemical ecology of poplars to a significant extent.

1.6. Research questions addressed in the thesis

Previous work on condensed tannins and salicinoids in poplars has contributed to the understanding of basic principles of direct defense and laid the groundwork for further studies of insect-plant interactions. However, within the genus *Populus* our current knowledge is widely restricted to one North American poplar (*Populus tremuloides*) and a handful of associated herbivore species. This thesis is part of a project aimed at establishing black poplar, an endangered European poplar species, as an alternate model organism to permit *in situ* studies of direct and indirect defenses in a natural environment. The overarching topic of this work was the phenolic-mediated direct defense of black poplar, while a parallel-project investigated the indirect defense mediated by volatile organic compounds (see Clavijo McCormick 2013). Both approaches included mechanistic studies of the respective defenses under laboratory conditions accompanied by field investigations to elucidate the possible consequences in nature. Field experiments were conducted in a natural reserve that accommodates the last large population of black poplar trees in Germany. At this site approximately 400 black poplar trees grow in their natural habitat, river floodplains, which are among the most endangered and biodiverse biotopes in Germany (Krause *et al.* 2011).

Three primary questions were addressed in this thesis. First, we wished to better understand the role of condensed tannins in the defense against generalist herbivores. Previous studies indicated that salicinoids had predominantly greater effects than condensed tannins in terms of anti-herbivore activity. However, many of these approaches were correlative and compared performance on genotypes differing in phenolics, but possibly also in other traits. The availability of condensed tannin over-expressing lines of *Populus tremula* x *tremuloides* together with unmodified wild type and GUS control lines gave us the opportunity to monitor the performance of generalist gypsy moth (*Lymantria dispar*) and forest tent (*Malacosoma disstria*) caterpillars on trees with an identical genetic background but different condensed tannin contents.

Since the results of this first study emphasized that salicinoids had more significant anti-herbivore properties than condensed tannins, we focused our subsequent efforts on this compound class. A careful survey of the literature revealed that there are major gaps in our knowledge of salicinoid toxicology in insect herbivores. Elucidating how salicinoids harm herbivores and how herbivores cope with them is required for understanding the molecular basis of their defensive function in poplars. Therefore we studied the physiological responses to salicinoid intoxication and explored the metabolic breakdown of three salicinoids in gypsy moth larvae.

Finally, we investigated the basic patterns of phenolic abundance in naturally growing black poplar trees. Since there was only little previous information about the phytochemistry of this species, a screening of phenolics and the development of routine analytical methods for their analysis was necessary. The phenolics which could be quantified were then investigated with respect to inducibility and seasonal or spatial patterns of distribution. The observations were carried out with mature trees under natural conditions to explore how phenolics influence the palatability of plant tissue for herbivores.

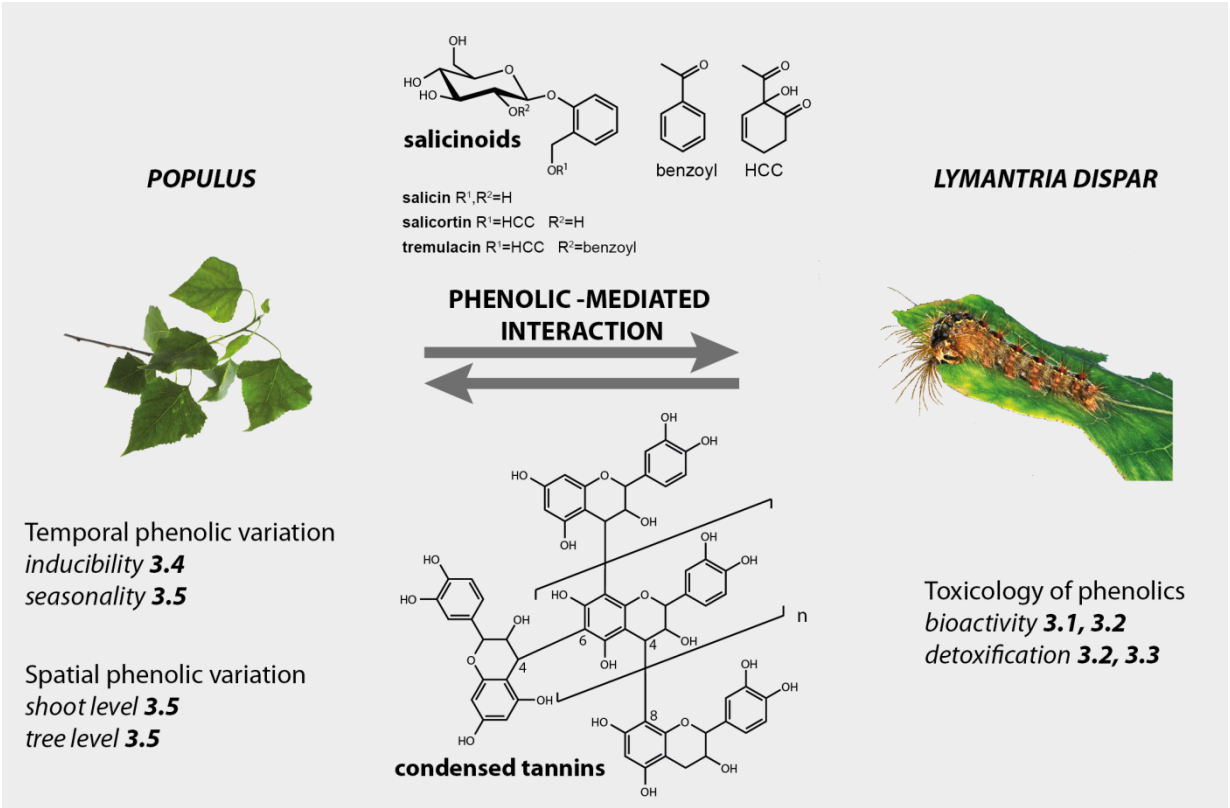

Fig. 2. Schematic representation of the phenolic-mediated interaction between poplar and the gypsy moth (*Lymantria dispar*). Structures show three exemplary salicinoids and condensed tannins, the two major classes of phenolics in poplars. Numbers indicate chapters where the according phenomenon is addressed.

2. Overview of the manuscripts

Manuscript I: Transgenic upregulation of the condensed tannin pathway in poplar leads to a dramatic shift in leaf palatability for two tree-feeding Lepidoptera

GA Boeckler, M Towns, SB Unsicker, RD Mellway, L Yip, I Hilke, J Gershenzon, CP Constabel

Published in the Journal of Chemical Ecology (2014), Volume 40, Issue 2, pp 150-158

Reprint License Number: 3561420767243

In this international collaboration, we explored the influence of condensed tannins on gypsy moth performance in a comparative approach with transgenic trees. Transgenic lines over-expressing the MYB 134 transcription factor had elevated levels of condensed tannins compared to wild-type and GUS controls. Foliage of each tree type was offered to gypsy moth and forest tent caterpillars in choice and performance experiments. Both species showed a significant preference for the transgenic trees with higher levels of condensed tannins. Likewise, gypsy moth caterpillars performed much better on the transgenic trees as indicated by lower mortality, faster development and higher pupal weights. Phytochemical analysis revealed that the enhanced performance on transgenic trees was likely due to a pleiotropic effect of the transformation that led to decreased salicinoid concentrations concomitant to increases in condensed tannins. The study underlines that of the two most abundant groups of phenolics in poplar, the salicinoids are far more potent defenses against insect herbivores.

Author contributions

Research conceived by: Constabel CP, Mellway RD, Unsicker SB, Boeckler GA (30 %)

Experiments designed by: Constabel CP, Unsicker SB, Boeckler GA (60 %)

Experiments conducted by: Towns M, Yip L, Hilke I, Unsicker SB, Boeckler GA (75 %)

Data analysis performed by: Towns M, Boeckler GA (80 %)

Manuscript written: Constabel CP, Gershenzon J, Boeckler GA (50 %)

Manuscript II: Phenolic glycosides of the Salicaceae and their role in herbivore defense

GA Boeckler, J Gershenzon, SB Unsicker

Published in: Phytochemistry 72 (2011) 1497-1509

Reprint License Number: 1982084

In this review article we highlight the chemical ecology of a group of salicin derivatives that are commonly termed “phenolic glycosides” or “salicylates” and are specific to the Salicaceae. We summarize the knowledge from the last decades about their occurrence and patterns of abundance. Moreover, we review the extensive research on the function of phenolic glycosides in shaping the insect plant interactions of the Salicaceae, including insights about induction, sequestration and mode of action. Finally, the name “salicinoid” is suggested instead of phenolic glycoside in order to avoid ambiguities that may arise from the traditional term.

Author contributions:

Literature search performed by: Unsicker SB, Gershenzon J, Boeckler GA (80 %)

Manuscript written by: Unsicker SB, Gershenzon J, Boeckler GA (80 %)

Manuscript III: Metabolism of salicinoids in the gypsy moth (*Lymantria dispar*)

GA Boeckler, C Paetz, J Gershenzon, SB Unsicker

In preparation for submission

In this study, we investigated the fecal detoxification products of salicinoids in the gypsy moth. In a first comparative experiment on diet artificially supplemented with three salicinoids, we identified five metabolites. Due to the modular composition of the administered salicinoids, each metabolite could be unambiguously assigned to a precursor moiety providing a deeper insight into the bioactivation and subsequent detoxification. We then studied if continuous exposure to dietary salicinoid constituents leads to a metabolic adaptation and alters the excretion rate of the five identified metabolites. Previous experience only affected the excretion rates of two novel glucose phosphate conjugates that were identified before. A final performance experiment showed that the major anti-herbivore activity resides in two widespread salicinoid moieties.

Author contributions

Research conceived by: Unsicker SB, Boeckler GA (90 %)

Experiments designed by: Unsicker SB, Boeckler GA (90 %)

Experiments conducted by: Unsicker SB, Paetz C, Boeckler GA (80 %)

Data analysis performed by: Paetz C, Boeckler GA (80 %)

Manuscript written by: Unsicker SB, C Paetz, Gershenzon J, Boeckler GA (80 %)

Manuscript IV: Gypsy moth caterpillar feeding has only a marginal impact on phenolic compounds in old-growth poplar

GA Boeckler, J Gershenzon, SB Unsicker

Published in the Journal of Chemical Ecology (2013), Volume 39, Issue 10, pp 1301-1312

Reprint License Number: 3561420645580

In this field study, we investigated the top-down effect of gypsy moth caterpillars on the phenolic content of black poplar. The major phenolic compounds in black poplar were identified and their response to experimental herbivory was investigated in two experiments; one was replicated on 20 trees of a black poplar population while the other experiment focused on a single tree. Analysis of phytohormones revealed an induction of defense-related signaling by the experimental herbivory. However, in neither of the two approaches was an induction of phenolics observed on a scale relevant for attacking herbivores.

Author contributions

Research conceived by: Unsicker SB, Boeckler GA (70 %)

Experiments designed by: Unsicker SB, Boeckler GA (70 %)

Experiments conducted by: Unsicker SB, Boeckler GA (80 %)

Data analysis performed by: Boeckler GA (100 %)

Manuscript written by: Unsicker SB, Gershenzon J, Boeckler GA (90 %)

Manuscript V: Spatiotemporal heterogeneity of phenolics in wild black poplar (*Populus nigra*)

GA Boeckler, J Gershenzon, SB Unsicker

In preparation for submission

The article presents data from a large scale field survey with the aim to investigate the annual variation of phenolic content in 20 mature black poplar trees under natural conditions. We also documented patterns of spatial distribution within the trees and within shoots. In addition, measures of herbivory and a late season leaf rust infestation were recorded and correlated to the levels of phenolic secondary metabolites. Phenolics levels were strongly dependent on the season and location within the plant. Major herbivory was restricted to the early vegetation period when the levels of most phenolics were still low, but was overall not correlated to any of the measured phenolics. In contrast, the flavan-3-ol catechol conferred resistance to leaf rust infestation, as the foliar concentration of this compound was negatively correlated to glucosamine, an indirect measure of fungal infestation.

Author contributions

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Experiments designed by: Unsicker SB, Boeckler GA (75 %)

Experiments conducted by: Unsicker SB, Boeckler GA (75 %)

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3. Manuscripts

3.1. Manuscript I: Transgenic upregulation of the condensed tannin pathway in poplar leads to a dramatic shift in leaf palatability for two tree-feeding Lepidoptera

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Transgenic upregulation of the condensed tannin pathway in poplar leads to a dramatic shift in leaf palatability for two tree-feeding Lepidoptera

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Abstract Transgenic hybrid aspen (*Populus tremula x tremuloides*) overexpressing the MYB134 tannin regulatory gene show dramatically enhanced condensed tannin (proanthocyanidin) levels, as well as shifts in other phenolic metabolites. A series of insect bioassays with forest tent caterpillars (*Malacosoma disstria*) and gypsy moth (*Lymantria dispar*) caterpillars was carried out to determine how this metabolic shift affects food preference and performance of generalist tree-feeding lepidopterans. Both species showed a distinct preference for the high-tannin MYB134 overexpressor plants, and *L. dispar* performance was enhanced relative to controls. *L. dispar* reached greater pupal weight and showed reduced time to pupation when reared on the MYB134 overexpressing poplar. These results were unexpected since enhanced condensed tannin levels were predicted to act as feeding deterrents. However, the data may be explained by the observed decrease in the salicinoids (phenolic glycosides) salicortin and tremulacin that accompanied the upregulation of the condensed tannins in the transgenics. We conclude that for these two lepidopteran species, condensed tannin levels are unlikely to be a major determinant of caterpillar food preference or performance. However, our experiments show that overexpression of a single regulatory gene in transgenic aspen can have a significant impact on herbivorous insects.

Keywords Plant-insect interaction · Plant defense · Phenolic glycoside · Defoliator · Genetic modification · Populus · Aspen · Salicinoid

Introduction

Phenolics are one of the most widespread families of plant metabolites, and have many ecological functions including herbivore defense. This group includes diverse compounds such as flavonols, anthocyanins, furanocoumarins, phenolic acids, and esters, as well as tannins. Tannins are high molecular weight polyphenolics functionally defined by their ability to precipitate proteins, and are grouped into two biosynthetic classes. The hydrolyzable tannins constitute the first class and are derived from galloyl glucose. The second class are the condensed tannins (CTs, also known as proanthocyanidins), which are products of the flavonoid pathway. Tannins are found throughout the plant kingdom, but in many herbaceous plants they accumulate primarily in the seed coat. By contrast, woody plants commonly contain tannins in vegetative tissues including roots, bark, and leaves. Their overall abundance in the leaves of forest trees, and early observations of a positive correlation between tannin levels and reduced insect

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herbivory (Feeny 1968) have led to the widespread expectation that tannins act as quantitative defenses against leaf-eating herbivores by precipitating dietary protein in insect guts. However, many other experiments have failed to demonstrate a general defensive role for CTs against insect herbivores (i.e., Ayres et al. 1997) or a significant impact of CTs on protein utilization (Barbehenn et al. 2009). The evidence for a direct impact of these compounds on folivorous insects is thus inconsistent (for a review, see Barbehenn and Constabel 2011).

Trees in the genus *Populus* contain high levels of CTs and other phenolic compounds (Constabel and Lindroth 2010). Furthermore, they often are dominant keystone species and are involved in a variety of ecological interactions, making them an excellent system for studying the role of phenolics in plant-insect interactions. Early work by Hwang and Lindroth (1997) demonstrated that *P. tremuloides* leaves can accumulate up to 25 % DW of CTs, in addition to high levels of other phenolics. The concentration of phenolics in poplar is extremely variable in natural populations and genotypes, which facilitated many ecological studies correlating phenolic content and insect performance (Hwang and Lindroth 1997; Whitham et al. 2008). Such studies generally report that the impact of even high CT levels on aspen defoliators is minimal (Hemming and Lindroth 1995). Rather, much of the defensive chemical potential of aspen leaves appears to reside in the phenolic glycosides, or salicinoids, a highly abundant group of phenolics in the Salicaceae (reviewed by Boeckler et al. 2011). The levels of phenolics and tannins cannot be easily manipulated in poplar foliage, and thus direct demonstrations of their ecological functions are lacking.

The elucidation of the *Populus trichocarpa* genome and the adoption of poplar as a model tree species provides new strategies for assessing the functions of phenolics, and has accelerated the discovery of genes relevant for their biosynthesis (Constabel and Lindroth 2010; Mellway et al. 2009). Together with the development of genetic transformation methods for *Populus*, this resource is facilitating the creation of transgenic poplars with modified gene expression. Genetically transformed poplar have permitted the direct testing of putative defense proteins and have generated novel insight into the functions of defense proteins and genes (Barbehenn et al. 2008; Wang and Constabel 2004). Recently, we identifed the poplar PtMYB134 gene as a key regulator of stress-induced CTs in *P. tremuloides*. This gene is induced by wounding and herbivory, and its overexpression in *P. tremula* x *tremuloides* leads to the activation of the flavonoid and the CT pathway, and results in the accumulation of up to 50-fold enhanced levels of CTs in transgenic aspen plants (Mellway et al. 2009). These MYB134 overexpressing plants present an opportunity for testing the biological effects of drastic changes in phenolic profiles in otherwise identical genetic backgrounds. Preliminary data suggested enhanced thrips susceptibility of MYB134-overexpressing lines of hybrid aspen (Mellway and Constabel 2009), while little effect on the specialist leaf beetle *Phratora vitellinae* was observed (Kosonen et al. 2012).

A major question is how tree-feeding caterpillars respond to enhanced CT levels in the transgenic foliage. Here, we report on insect bioassays performed with the forest tent caterpillar (*Malacosoma disstria*) and gypsy moth (*Lymantria dispar*). The former is an oligophagous lepidopteran that typically feeds on *P. tremuloides* in the boreal forest of Canada. The latter is a polyphagous herbivore that feeds on numerous woody plant species, such as oak, poplar, and willow, and which can cause complete defoliation in monocultural stands. Our results demonstrate that MYB134 overexpression in hybrid aspen leads to a dramatic shift in feeding preference and performance of these two species of Lepidoptera, but that high CT levels may not be the primary cause of this shift.

Methods and Materials

Plant Material The MYB134 overexpressing transgenic poplars have been described previously (Mellway et al. 2009). For these experiments, we used transgenics that had been created in the *P. tremula X P. tremuloides* INRA 353–38 hybrid. Control plants were either untransformed ("wild-type") or plants overexpressing the GUS reporter gene (Datla et al. 1992). GUS overexpressors were previously shown to have normal levels of phenolics and tannins (Mellway et al. 2009). None of the MYB overexpressors showed any growth abnormalities under greenhouse conditions, and no anomalous effects due the plant transformation procedure itself have ever been observed in our laboratory. Several independently transformed MYB134 and GUS control lines were used in all experiments. The MYB134 overexpressing lines are referred to as MYB line-1 to MYB line-5, corresponding to independent transformants MYB134-46, MYB134-54, MYB134-61, MYB134-62, and MYB134-64, respectively. Control plants are referred to as control-1 to control-3, corresponding to wild-type, GUS-18, and GUS-71 plants, respectively. Both transgenic and control plants were maintained and micropropagated as *in vitro* shoot cultures on half-strength Murashige-Skoog or Woody Plant Medium (Caisson Laboratories, Logan, UT, USA) supplemented with 0.01 ug/l indole butyric acid. Clonal copies of these lines were shipped to the Max-Planck Institute for Chemical Ecology, Jena, Germany, for the *Lymantria dispar* caterpillar experiments. Prior to being moved into a greenhouse or growth chambers, *in vitro* plantlets were transplanted into sterilized soil in small containers and acclimated under high humidity. Plants were maintained under long-day conditions (16 h light) and kept well-watered. Plants used for experiments in Victoria were typically 8–10 wk-old and had 20–25 leaves.

Experimental trees in Jena were typically 7 mo-old and had 70–100 leaves.

Chemical Analyses Sucrose, glucose, and fructose levels were measured using an enzymatic assay as described by Campbell et al. (1999), with leaf extracts prepared from freeze-dried leaf tissue. Starch was assayed by the same method, but first enzymatically converted to glucose (Hendrix et al. 1993). Phytochemical analysis of leaf material was performed with extracts of leaves harvested during the gypsy moth performance experiment. The availability of transgenic foliage was a limiting factor in this experiment, and the leaves offered to the caterpillars could not be exchanged more than once every 5–7 d. Therefore, we analyzed the foliage after the experiment to show that the phenotypic patterns reported in Mellway et al. (2009) persisted within this time frame. Five replicate leaves per tree line were sampled after they had been offered to the caterpillars. Leaf material was flash-frozen in liquid nitrogen, freeze-dried, and ground to a fine powder. CTs were assayed using the BuHCl method (Porter et al. 1986) using purified aspen CT as the standard as previously described (Boeckler et al. 2013). For salicinoid analysis, 10 mg of the dried powder was extracted twice with 1 ml methanol. In the first extraction step, 0.8 mg/ml of phenyl-β-glycopyranoside (Sigma) was added as an internal standard. The combined extracts were analyzed by HPLC as previously described (Boeckler et al. 2013). C/N ratios of 15–18 mg of the dried leaf samples were determined with a vario EL elemental analyzer (Elementar Analysensysteme GmbH, Hanau, Germany).

Malacosoma disstria Experiments Forest tent caterpillar (*Malacosoma disstria*) egg masses, collected in the field near Prince George, British Columbia, were obtained from Dr. Jens Roland (University of Alberta, Edmonton, Canada) and kept for up to 4 mo at 4 ° C until used. Spumaline was scraped from egg masses with a sterilized scalpel, and the egg masses were surface-sterilized using with 1 % commercial bleach for 3 min, followed by rinsing in ddH_2O for 5 min. Egg masses were placed on artificial diet (Bio-Serv, Frenchtown, NJ, USA) and neonate larvae were maintained at 20 °C and ambient light in the laboratory. Second-instar caterpillars of the same age and from the same cohort were selected for each experiment. These were typically one wk-old. For *M. disstria* preference assays, one of three high-tannin MYB plants (MYB line-1, MYB line-3 or MYB line-4) was paired with a matched control (control-1) plant. The plants were approximately 2 m in height with sufficient foliage for the entire series of experiments. Leaves of matching age and size were harvested, rinsed with distilled water, and leaf disks (16 mm diam) were cut from the leaves using a cork borer. Ten leaf disks from one MYB line and one control line were arranged around the perimeter of a Petri-dish (85 mm diam) lined with moistened Whatman #1 filter paper. MYB and control leaf disks were alternated. Five second instars were placed onto the filter paper in the center of the dish. Four replicate arenas of each pair were established. The dishes were sealed with Parafilm and maintained in the laboratory under ambient light conditions and room temperature. After 2 d of feeding, leaf disk area consumed was determined. Leaf disks were first photocopied onto acetate sheets, and the area consumed was traced onto a second acetate sheet using a black pen. All disks from one petri plate were traced onto one sheet, and the total area consumed of each plant line per dish was measured as a pool. Area was measured using a leaf area meter (Licor 3100, Lincoln, NE, USA).

For *M. disstria* performance tests, 16 mm diam leaf disks also were used, as the poplar leaves were too large to be easily managed. Four disks were placed in the center of 85 mm Petri plates, on Whatman #1 filters previously moistened with 1 ml of sterile ddH_2O. Five one-wk-old larvae were placed in the center of the dish among the leaf disks. Any larvae that did not survive the first 24 h after transfer were removed and replaced. Dishes were sealed with Parafilm, and water was replaced as necessary. Foliage consumed was calculated on a unit area basis. The entire experiment was repeated on separate days.

Lymantria dispar Experiments In order to test if another generalist tree-feeding lepidopteran would also prefer poplar MYB overexpressor plants, preference and performance tests were established for gypsy moth (*L. dispar*) caterpillars. Clonal replicates of MYB and control transgenic lines were shipped to Jena, Germany, where they were propagated *in vitro* and acclimated to growth chambers. Gypsy moth preference and performance experiments initially were carried out with three MYB lines (line-1, line-3, and line-5) and three controls (control-1, control-2, and control-3). However, subsequent phytochemical analyses showed that MYB line-3 had lost its high CT phenotype, also observed by Kosonen et al. (2012), and insect data from this line therefore were not used in the analysis. Gypsy moth (*L. dispar*) caterpillars were hatched from egg clutches provided by Melody Keena (United States Department of Agriculture, Hamden, CT, USA.) and reared in a climate chamber (day: night, photo period 16:8, temperature 24 °C:18 °C, humidity 60 %) on artificial gypsy moth diet (MP Biomedicals, Eschwege, Germany) until the onset of the experiments.

Lymantria dispar feeding preference assays were conducted in modified 90 mm Petri-dishes. In the bottom of the Petri-dish, eight equidistant holes were drilled in a circular arrangement 3 cm from the middle to allow drawing pins to protrude into the dish. Moist filter paper was placed on the bottom of the arena, and pins were pierced through the filter paper. Four leaf discs (16 mm diam) of each of two tree lines were alternately fastened with the drawing pins. A 4^{th} instar *L. dispar* (starved for 24 h) was placed in the center of the arena and allowed to feed on the leaf discs for 3 h. During the experiment, the arenas

were kept under controlled conditions in a climate chamber (constant light, 24 °C, 70 % humidity). To score results, the larvae were removed, and the leaf discs were photographed. The consumed leaf area was reconstructed and measured using Adobe Photoshop CS4 (Adobe, San Jose, CA, USA).

For performance assays, *L. dispar* larvae were reared on foliage of MYB overexpressing and controls plants in a climate chamber maintained at the same conditions as for the preference assay. Care was taken to offer leaves of similar age among all tree lines. To facilitate data acquisition, the experiment was divided into two phases. For the first 18 d (Phase 1), caterpillars were reared in groups. After d 18 (Phase 2), caterpillars were separated and reared individually until pupation. To begin the experiment, ten groups of five neonate larvae were transferred into 145 mm Petri-dishes and provided freshly excised leaves. Leaf petioles were kept in plastic tubes filled with distilled water to avoid desiccation. Water was refilled when necessary, and the leaves were changed every 6 d or as soon as signs of wilting were observed. Until d 5, dead larvae were replaced by healthy individuals of the same cohort maintained on artificial diet. In Phase 1 of the performance assays, caterpillars were weighed in groups on day 1, 6, 12, and 18. Survival was recorded daily. On d 18, when caterpillars were typically in their 2nd or 3rd instar, Phase 2 measurements started, and ten caterpillars of each tree line were randomly selected to be reared individually until pupation. Individual caterpillars or pupae were weighed in 4–8 day intervals when the foliage was exchanged and the date of pupation was recorded. Adults were allowed to hatch and the sex was determined.

Leaf consumption during Phase 1 of *L. dispar* performance experiments was too low to be determined accurately, as the leaves gained weight from water uptake. Therefore, the leaves were weighed upon excision and photographed before caterpillar feeding for leaf area determination as described above. When old leaves were exchanged for new leaves, they were photographed again to determine caterpillar-related leaf area loss. The biomass consumed by the caterpillars then was calculated on the basis of the initial leaf area: leaf weight ratio. In Phase 2 of the experiments, the consumption was calculated as the difference of the leaf weight before and after the experiments.

Statistical Analysis For statistical testing we used the statistical software R 2.15.0 (R Development Core Team, http://www.R-project.org). All data were checked for statistical assumptions such as normal distribution and homoscedasticity. In preference assays, the consumed area of both offered tree lines was compared using a paired *t*-test after normality was confirmed by a Shapiro-Wilk test. Larval survival was statistically investigated with a parametric Kaplan-Meier survival analysis with log-logistic error distribution. The recording of caterpillar survival for this analysis started at d 7 of the performance experiment and ended when the last caterpillar pupated. Censoring was applied when caterpillars pupated or left the experiment alive after the end of Phase 1. After running a basic model treating all tree lines independently, statistical significance was investigated through model reduction. Tree lines with similar survival were combined stepwise, and this reduced model was compared with the previous until the minimum adequate model was achieved. Caterpillar weight gain was log-transformed and tested as fixed effect against the null model using a linear mixed effect model with tree line and sampling date as random effects. Both experimental phases were tested separately. Due to the limited number of replicates, the influence of sex could not be accounted for, and it was assumed that both sexes developed equally until pupation. Therefore, only data points before the first pupation on d 37 were included in analysis of Phase 2. Phytochemical data were analyzed on the basis of a one-way ANOVA with the tree line as factor. The Tukey HSD function was used as a *post-hoc* test.

Results

Plant Characteristics Our previous work had indicated that the MYB134 overexpressor *P. tremula* x *tremuloides* plants had significantly higher foliar condensed tannin (CT) levels, but showed only minor alterations of the other major flavonoids and phenylpropanoids (Mellway et al. 2009). Microarray analysis suggested that genes in other metabolic pathways generally were not affected by MYB134 overexpression (R. Mellway, A. Seguin, and C.P. Constabel, unpublished data). We also checked if the content of major sugars (glucose, fructrose, and sucrose), as well as starch, might be altered in representative MYB lines. However, we found no significant deviation from the control lines for any of the sugars or starch (Table 1).

Malacosoma disstria Experiments In preference tests carried out in Petri-dish arenas, *M. disstria* larvae showed a strong preference for the MYB leaf disks, feeding almost exclusively on these genotypes. Up to 40 % of the leaf disk area was consumed from the transgenic disks in a 2-day experiment, while the consumption of control disks was much lower than

Table 1 Soluble carbohydrate analysis of MYB overexpressor and control poplars*

plant line	fructose	glucose	sucrose	starch
line 1	17.4 (1.4)	28.9 (2.2)	39.9 (0.1)	5.3 (0.5)
line 4	18.5 (0.7)	27.6 (2.6)	32.7 (1.6)	6.5 (0.5)
con-1	16.8 (2.8)	28.3 (3.3)	37.6 (0.6)	5.2 (0.2)
con-3	15.5 (0.3)	28.3 (1.1)	42.1 (1.6)	5.2 (0.2)

*Values show mg/g DW with standard deviation in parenthesis. None of the sugars was significantly different between the plant lines.

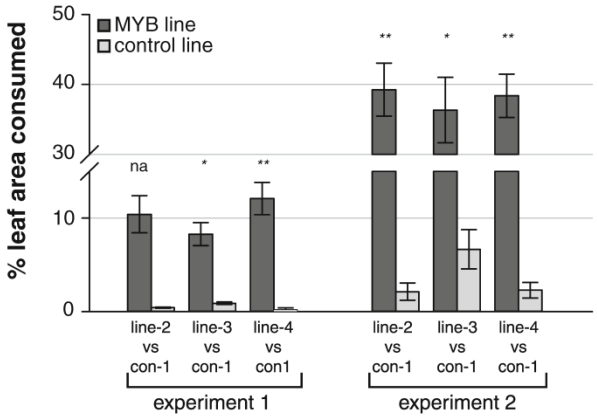

Fig. 1 *Malacosoma disstra* caterpillar preference in two-choice cafeteria assays. First instar larvae were allowed to feed for 2 d in Petri-dish arenas with alternating control and MYB plant disks. Leaf area consumed was measured after 48 h. Experiments were conducted with seven 7-d-old larvae (left panel), or eight 8-d-old larvae (right panel). Bars represent means ± SE. Asterisks indicate significant differences within paired t-tests (***$P<0.001$, ** $P<0.01$, * $P<0.05$, na=not assessed)

10 % (Fig. 1). Overall rates of consumption differed between experiments due to different ages of larvae for each experiment. The preference for MYB foliage was independent of which overexpressor line was used, and this also was observed when MYB lines were paired with GUS overexpressor lines (data not shown). Therefore, despite the greatly enhanced levels of tannins in transgenic MYB plants, these were clearly preferred.

The consumption of control foliage by *M. disstria* in the preference assays was so low that the feasibility of running long-term performance assays was questionable. Therefore, a preliminary experiment was initiated to test if the survivorship on control leaves was sufficiently high. Second instar *M. disstria* caterpillars were offered leaf disks of either MYB or control trees and monitored. The larvae did not feed as readily on the control disks as they did on the transgenic disks, which led to high mortality rates (approximately 50 % in 6 days; data not shown). This made long-term performance experiments with *M. disstria* unfeasible.

Lymantria dispar Experiments When the MYB line-1 and line-2 were offered against controls in choice assays, *L. dispar* caterpillars also showed a consistent and significant preference for the MYB lines (Fig. 2). The caterpillars consumed larger portions of the foliage of line-1 and line-5 regardless of the comparison lines, and this effect was statistically significant in most of the pairings. MYB line-1 foliage appeared to be the most preferred, as it showed the greatest differential to controls and was also preferred over MYB line-5 (Fig. 2, left panel). These results confirmed that MYB134 overexpression is associated with increased feeding preference for *L. dispar* as it had for *M. disstria*.

Despite their strong preference for the MYB plants, gypsy moth larvae fed readily on control foliage (GUS or WT) and therefore survived much longer on control foliage than *M. disstria*. Survivorship analysis showed that caterpillar mortality was significantly influenced by the type of plant line offered (Fig. 3). Between 60 % and 80 % of the caterpillars fed with MYB foliage reached the adult stage, compared to less than 25 % in the controls. When a model simplification was applied to analyze which survival curves differed significantly, larvae from the five lines clustered in four groups, each with similar survival: con-2, group con-1 / con-3, MYB line-1, and MYB line-5. Con-2 larvae had a significantly higher survival than con1/ con-3 larvae ($P=0.026$), but a significantly lower survival than larvae on line-1 ($P=0.010$) and line-5 ($P<0.001$). Overall, there was a much higher survival rate (> 0.6) on all the MYB lines compared to the control groups (Fig. 3).

In addition to survival, other fitness parameters (caterpillar weight gain, time until pupation, and consumed biomass) were recorded during the performance experiment. Caterpillar weight gain was significantly influenced by the

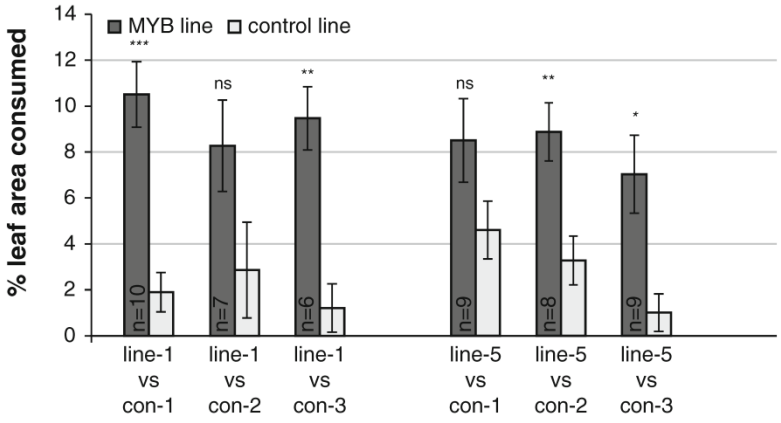

Fig. 2 *Lymantria dispar* caterpillar preference in two-choice cafeteria assays. In each assay, four leaf discs of the respective MYB line (*dark bars*) were offered together with four leaf discs of a control line (*light bars*) to a starved 4th instar caterpillar. After 3 h, the consumed leaf area of both types was determined. Bars represent means ± SE. Asterisks indicate significant differences within paired t-tests (***$P<0.001$, ** $P<0.01$, * $P<0.05$, ns=non-significant)

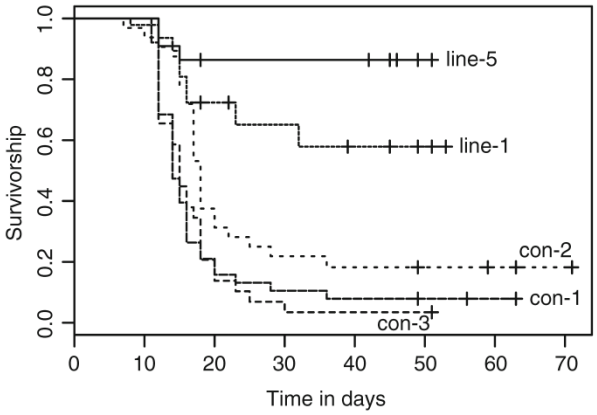

Fig. 3 Kaplan-Meyer survivorship curve for *Lymantria dispar* caterpillars in the performance experiment. Caterpillars were reared from eclosion until pupation on the foliage of one of the shown tree lines. Survival was recorded from d 7 of the performance experiment until d 72, when the last caterpillar pupated. Crosses indicate

tree line in phase 1 (P=0.014) and phase 2 (P<0.001). Both female and male larvae feeding on MYB line-1 and line-5 gained more weight over time, than larvae on controls (Fig. 4). In addition, pupal parameters and food conversion varied with the tree line, but these effects could not be tested statistically. Generally caterpillars reared on MYB lines showed a trend to faster pupation, higher pupal weights, and better food conversion than larvae reared on control lines (see Supplemental Fig. S1c). Survival on control-3 was so low that only one male caterpillar pupated, and thus no performance data are shown for this line.

Phytochemical Analyses in L. dispar Experiments We performed phytochemical analysis on foliage used for *L. dispar* performance assays to confirm that the MYB phenotype had remained unchanged in these plants. HPLC and BuOH/HCl analysis showed that foliage of both MYB lines tested contained approximately two times greater concentrations of CTs and its biosynthetic precursor, catechin, and this effect was statistically significant (both P<0.001, Fig. 5). We also measured the major salicinoids and conducted C/N analyses, since these two factors may influence gypsy moth development, and the salicinoids are known to be impacted by MYB 134 overexpression (Mellway et al. 2009). Salicortin and tremulacin occurred in much lower amounts in MYB lines (both P<0.001, Fig. 6), while salicin levels were not significantly different between MYB lines and controls (P=0.203). In addition, MYB line-1 had a slightly increased C/N ratio compared to all other lines (P<0.001, Fig. 6).

Discussion

Genetically transformed plants can be powerful tools for the functional analysis of plants and have provided direct demon-

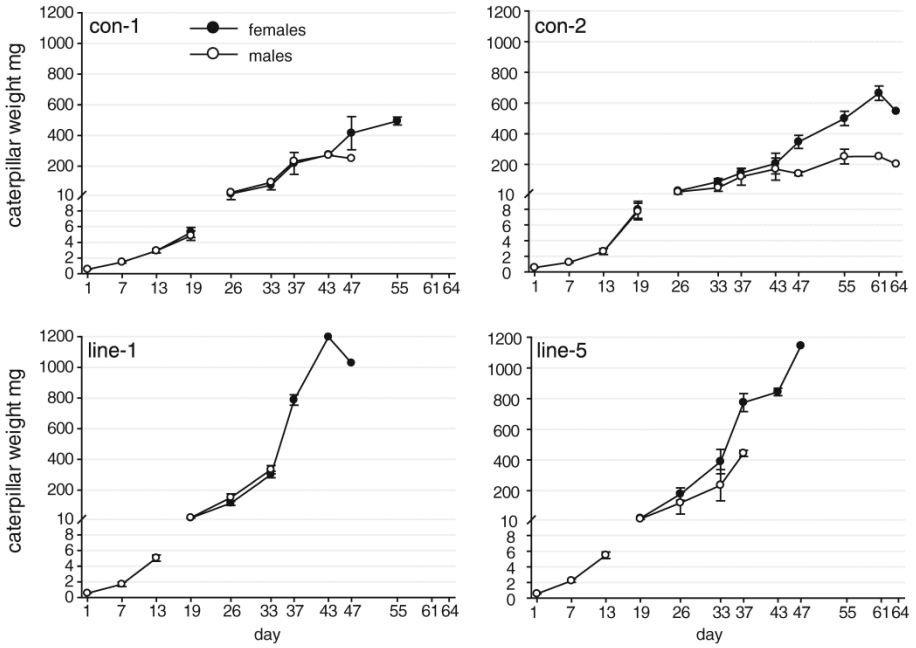

Fig. 4 Weight gain of *Lymantria dispar* caterpillars in the performance experiment. The first three data points represent averaged weights recorded during phase 1 of the experiment, when caterpillars were reared in groups and sexes were not discriminated. From d 19 onwards, data points show averages of individual larvae. Pupae are not included. Error bars show SE. Note split scaling on vertical axis

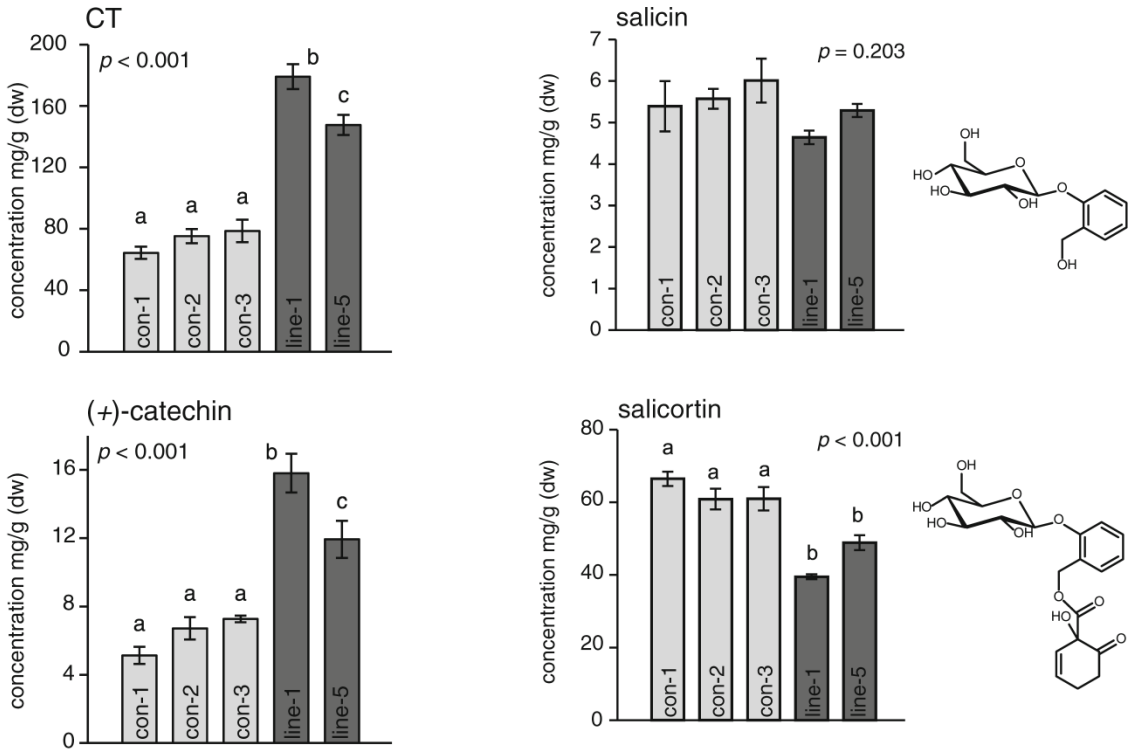

Fig. 5 Condensed tannin (CT) and catechin of MYB line and control foliage from *Lymantria dispar* larvae performance experiment. Bars show means ± SE, N=5. P values refer to ANOVA with the tree line as independent and the concentration/ratio as continuous variable. Letters indicate result of Tukey HSD *post hoc* test, P<0.001

strations of the efficacy of plant defenses against herbivorous insects (Rasmann and Agrawal 2009). Transgenic *Populus* are an excellent experimental system for analysis of ecologically relevant genes, in particular related to phenolic metabolism (Constabel and Lindroth 2010). Here, we analyzed the impact of overexpressing the MYB134 tannin regulatory gene in poplar on *M. disstria* and *L. dispar*, generalist insect herbivores that readily feed on *P. tremuloides* and related *Populus* species. Our data show that overexpression of this gene caused a dramatic increase in palatability of foliage, and that larvae consuming MYB 134-overexpressing (high-tannin) foliage showed better survival and performance compared to larvae feeding on control leaves. We observed the same

Fig. 6 Salicinoid content and C/N ratio of leaves from *Lymantria dispar* ▶ larvae performance experiment. Bars show means ± SE, n=5. P values refer to ANOVA with the tree line as independent and C/N ratio as continuous variable. Letters indicate result of Tukey HSD *post hoc* test, P<0.001

dramatic shift in preference and performance in experiments carried out with two different lepidopteran species and with plants grown and tested in separate laboratories.

Both sets of experiments reported here compared clonal *P. tremula x tremuloides* tree lines overexpressing the MYB134 tannin-regulatory gene (MYB lines) with GUS-overexpressing or wild-type plants (controls). The effects of MYB134 over-expression on the CT phenotype has been described previously (Mellway et al. 2009), and was additionally validated here at the end of the *L. dispar* performance assay using leaves previously offered to the caterpillars. Transgenic line-1 and line-5 accumulated CTs to approximately 180 mg/gDW, significantly higher concentrations than those observed in control plants. The foliar CT concentrations of these MYB lines are high for young greenhouse-grown saplings; however, CT levels in this range are reported for mature outdoor-grown trees (Donaldson et al. 2006). Catechin, a precursor in CT biosynthesis, also was produced in higher amounts in the MYB lines. Thus, the phenotype in these transgenic leaves was consistent with our previous report.

Contrary to our expectations and to the common assumption that CTs act as general defensive phytochemicals, *M. disstria* and *L. dispar* showed a preference for MYB foliage despite the elevated tannins levels, and *L. dispar* also developed better on high-tannin plants. We performed additional phytochemical analyses to test if changes in the salicinoids (salicortin, tremulacin, and salicin) or C/N ratios could explain our data. These parameters are important predictors for gypsy moth performance on *P. tremuloides* (Hemming and Lindroth 1995). As observed previously by Mellway et al. (2009), the MYB lines had approximately 50 % lower concentrations of salicortin and tremulacin. This reduction in salicinoids seems most likely to explain our insect data, as these compounds have been shown to have adverse effects on several other lepidopteran species including *Papilio glaucus glaucus* (Scriber et al. 1989) and *Operophtera brumata* (Ruuhola et al. 2001), as well as *L. dispar* and *M. disstria* (Hemming and Lindroth 1995; Hwang and Lindroth 1997; Osier et al. 2000). Our data showing enhanced growth and reduced mortality on the transgenic MYB foliage in performance tests are thus consistent with the toxic effects of the salicinoids. Although the mechanism of toxicity is not fully understood, it has been related to a spontaneous or enzyme catalyzed breakdown of the salicinoids and the subsequent formation of electrophiles (reviewed in Boeckler et al. 2011). By contrast, the possible repellent effects of salicinoids on caterpillars suggested by our preference data have not yet been systemically investigated, and it is unclear if these are pre- or post-ingestive.

Despite the concomitant alterations of salicinoid levels with CTs in the transgenic lines, the preference experiments suggest that high tannin levels do not deter *M. disstria* and *L. dispar* from feeding. A broad evaluation of the literature suggests that the role of CTs as defense molecules against lepidopteran herbivores is complex (reviewed in Barbehenn and Constabel 2011). For example, Pearse (2011) found condensed tannin contents of different oak species to be negatively correlated to tussock moth survival. By contrast, direct bioassays showed that supplementation of sugar maple leaf disks with 15 % DW of CTs had no effect on growth rates or net growth efficiencies of *L. dispar* (Barbehenn et al. 2009). Both gypsy moth caterpillars and forest tent caterpillars used here are polyphagous and use a broad spectrum of deciduous trees and shrubs as host plants, and can even be found feeding on conifers and herbaceous species during outbreaks. Thus, the *Populus* CTs may not be effective defenses against tree-feeding Lepidoptera that are routinely exposed to and likely adapted to tannins and other woody plant polyphenols. By contrast, tannins may also prevent non-tree-feeding pests from expanding their ranges and moving onto tree hosts. This could be tested in the future by generating transgenic poplar that have reduced tannin levels and exposing these to naturally occurring pests.

Correlative studies on oak herbivore communities have shown that CTs negatively correlate with abundance of specialist insects more than that of generalists (Forkner et al. 2004). This suggests that CTs are more effective as defenses against specialist rather than generalist herbivores. Coleopteran larvae also are more likely to be affected by CTs than lepidopterans (Ayres et al. 1997), consistent with differences in gut pH (Barbehenn and Constabel 2011); future work with high-tannin poplar should consider coleopteran herbivores as well.

The inverse relationship of CT levels with reduced salicinoid concentrations that appears to underlie the observed shift in leaf palatability of the transgenics is intriguing. We speculate that this is due to competition for biosynthetic precursors within the phenylpropanoid pathway, although we cannot rule out more direct regulatory mechanisms. Trade-offs in resource allocation including defense chemicals often are predicted and have been observed between CT and salicinoid levels in *P. tremuloides* under resource-limiting conditions and competition (Donaldson et al. 2006; Osier and Lindroth 2006). Given the generally high phenolic content of aspen foliage (up to 30 % DW), the idea that precursor availability or other resources can limit overall investment in phenolic secondary metabolites is intuitively appealing. If this is generally the case, it would complicate the manipulation of phenolic metabolites. Generally, phenolic pathways appear to be plastic and particularly prone to unexpected effects of transgenic manipulations. Several earlier studies have reported altered phenolic glycoside concentrations in response to genetic modifications of the lignin pathway (Coleman et al. 2008; Ranocha et al. 2002). Together with the current work, such studies suggest that it could be difficult to alter specific phenolic constituents of poplar without causing non-target effects on other aspects of phenolic metabolism, and that careful screening of the transgenic phenotypes is essential.

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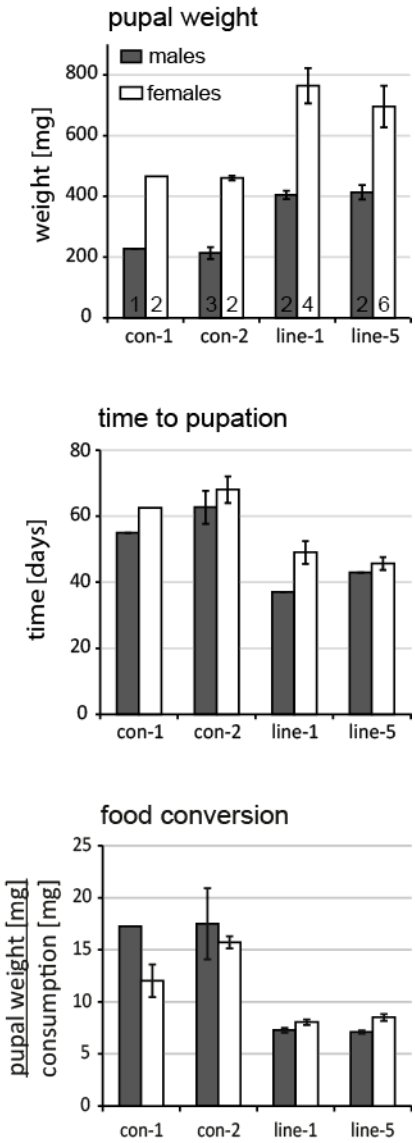
Appendix



Supplemental Fig. S1 (pupal parameters). Pupal weight, time to pupation and ratio of pupal weight to total consumption of *Lymantria dispar* larvae (performance experiment). Pupal weights were determined right after pupation as pupae tended to lose weight during metamorphosis. Number of replicates are shown in columns of the pupal weight graph and apply to all graphs. Means ±SE. No statistical analysis was possible due to low number of replicates

3.2. Manuscript II: Phenolic glycosides of the Salicaceae and their role as anti-herbivore defenses

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Review

Phenolic glycosides of the Salicaceae and their role as anti-herbivore defenses

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ABSTRACT

Since the 19th century the phytochemistry of the Salicaceae has been systematically investigated, initially for pharmaceutical and later for ecological reasons. The result of these efforts is a rich knowledge about the phenolic components, especially a series of glycosylated and esterified derivatives of salicyl alcohol known as "phenolic glycosides". These substances have received extensive attention with regard to their part in plant–herbivore interactions. The negative impact of phenolic glycosides on the performance of many generalist herbivores has been reported in numerous studies. Other more specialized feeders are less susceptible and have even been reported to sequester phenolic glycosides for their own defense. In this review, we attempt to summarize our current knowledge about the role of phenolic glycosides in mediating plant–herbivore interactions. As background, we first review what is known about their basic chemistry and occurrence in plants.

Contents

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1. Introduction

Though consisting of barely more than 20 compounds, the phenolic glycosides of the Salicaceae have received disproportionate attention from both phytochemists and chemical ecologists. This can be attributed, at least in part, to their pharmaceutical importance as anti-inflammatory agents which has been known for over 100 years. Other reasons for the great interest are that phenolic glycosides are some of the most abundant secondary metabolites known in plant tissues, and have been identified as important factors in many plant–herbivore studies conducted on poplar, aspen and willows where they have been implicated as toxins and deterrents to a number of insect and mammalian herbivore species.

Here, we summarize the role of phenolic glycosides as anti–herbivore defenses in the Salicaceae. These substances have also been covered in review articles on the secondary metabolites of the Salicaceae (e.g., Chen et al., 2009; Pierpoint, 1994) but have not yet been treated in systematic fashion. Thus, we also describe other aspects of their chemistry and occurrence in plants, including structural properties, methods of chemical analysis, biosynthetic pathways and patterns of genetic, developmental and seasonal variation. However, we do not cover the extensive literature on how phenolic glycosides are influenced by abiotic factors including light, nutrients, water regime and global change variables and their correlation with plant growth (e.g., Harding et al., 2009).

2. Chemical properties of phenolic glycosides

2.1. Structure and terminology

In a broad sense, the word "phenolic glycoside" refers to any molecule containing a sugar unit bound to a phenol aglycone. This description encompasses a vast number of secondary metabolites with only distant chemical or biosynthetic relationships. However, historically the term phenolic glycosides (PGs) has come to be applied just to compounds made up of a core structure consisting of salicyl alcohol and β-D-glucopyranose moieties, with an ether linkage between the phenolic hydroxyl group and the anomeric C-atom of the glucose. This definition will be followed here.

The simplest PG is salicin or D-(−)-salicin (Fig. 1) and hence PGs might best be referred to as "salicinoids". Salicin can be found in many Salicaceae species and is a basic element of the approximately 20 other more complex PGs formed by the esterification of one or more hydroxyl groups (that of the salicyl alcohol function or those of the glucose moiety) with various organic acids. A CAS Scifinder (http://www.cas.org/products/sfacad/index.html) structure search gave 22 compounds (Fig. 1) that matched the structural requirement of a salicin core structure. A few known compounds, namely nigracin, populoside A and salireposide, contain gentisyl alcohol (with an additional free hydroxyl group *para* to the phenolic hydroxyl group of salicin) instead of salicyl alcohol as their basic aglycone (Fig. 1), and these are also regarded as PGs. In contrast, glycosylated derivatives of salicylic acid, rather than salicylic alcohol (e.g., trichocarpin), and other glycosylated phenylpropanoids (e.g., vimalin), phenylethanoids (e.g., salidroside) and benzenoids are not included here even though they may also be specific to the Salicaceae. The esterification of the salicin sub-structure in complex PGs usually occurs at the primary alcohol function of the salicyl alcohol moiety and at the positions 2′ and 6′ of the glucose moiety (Fig. 1) with variable organic acids, commonly benzoic acid and/or 1-hydroxy-6-oxocyclohex-2-en-1-carboxylic acid (HCC), as in tremulacin. Conjugation with these aromatic or aliphatic acids attenuates the hydrophilic character of the salicin core decreasing its water solubility.

The ester bonds of complex PGs are susceptible to chemical and enzymatic hydrolysis, and so these molecules will break down to salicin if not stored appropriately (Lindroth and Pajutee, 1987). Salicin is non-reactive at room temperature, but it has been reported to be light sensitive (Hilden and Morris, 2003) and can be hydrolyzed to yield glucose and salicyl alcohol enzymatically or with dilute acid (Pinto and Diogo, 2008). In contrast to other phenolic compounds in the Salicaceae, such as flavonoids or condensed tannins, most PGs cannot undergo typical anti-oxidative reactions due to the lack of free phenol groups (Zhang et al., 2006) or electron rich double bonds. However, metabolic breakdown of HCC-containing PGs leads to the formation of highly oxidative species (see Section 6).

2.2. Chemical analysis of phenolic glycosides

The preferred way of sampling plant tissue for PG analysis is immediate flash-freezing followed by freeze-drying. Care should be taken to prevent thawing of samples before dryness since hydrolytic reactions in thawed tissue can cause a significant loss of complex PGs (Lindroth and Koss, 1996; Orians, 1995). Alternatively, fresh samples can be vacuum dried without PG loss if they can be processed rapidly (Lindroth and Koss, 1996; Orians, 1995). Dried samples are usually ground and extracted with MeOH or aqueous MeOH. In the latter case, samples should be analyzed promptly to avoid ester hydrolysis. Many authors enhance the extraction process by ultra-sonication (e.g., Förster et al., 2010) or repeatedly extract the tissue to maximize PG recovery.

PG-containing extracts have been analyzed by GC, TLC and HPLC systems. However, GC analysis requires silylation to form volatile derivatives and is therefore rarely used any more. The most frequently reported method is HPLC with gradient elution and UV detection. H_2O spiked with acid and MeOH or acetonitrile are common mobile phases employed with reversed phase C_{18} columns. Standard diode-array detectors are capable of sensitive detection of PGs. The UV chromophore is the aromatic π-electron system of the salicyl alcohol or other aromatic substituents and exhibits the typical absorption spectrum of the benzene ring with three bands caused by π–π^* electron transitions. Many authors use the α-band at about 280 nm which is least sensitive, but most specific for this chromophore. In recent years, the use of LC/MS-systems, typically in the negative ionization mode has become more common for PG analysis.

The unequivocal identification of PGs can only be realized by comparing their retention times to those of authentic standards. Pure standards are also needed to generate curves for absolute quantification. As most PGs are not commercially available, laborious purification from bark or leaves is often necessary. Protocols for purification have been published (Si et al., 2009b; Zhang et al., 2006).

3. Occurrence, patterns of variation and dynamics

Compared to other secondary metabolites, PGs can be very abundant in species of the Salicaceae, and concentrations of up to 30% of plant dry weight have been reported (Donaldson et al., 2006b). However, some species within the Salicaceae do not contain PGs at all (Palo, 1984). The distribution of these compounds within species shows extensive variation with genotype, season and stage of development, and variation among organs is also likely, but less well studied. PGs are commonly found in shoot tissues such as leaves, petioles, internodes, flowers and bark, but neither wood nor root tissue has been investigated thoroughly.

3.1. Genotypic variation of PGs

To date, the more than 20 different PGs described have been found in variable concentrations in members of the Salicaceae. Although some compounds, such as salicortin and salicin, are very widespread, others occur in only a few species (Table 1). Thus,

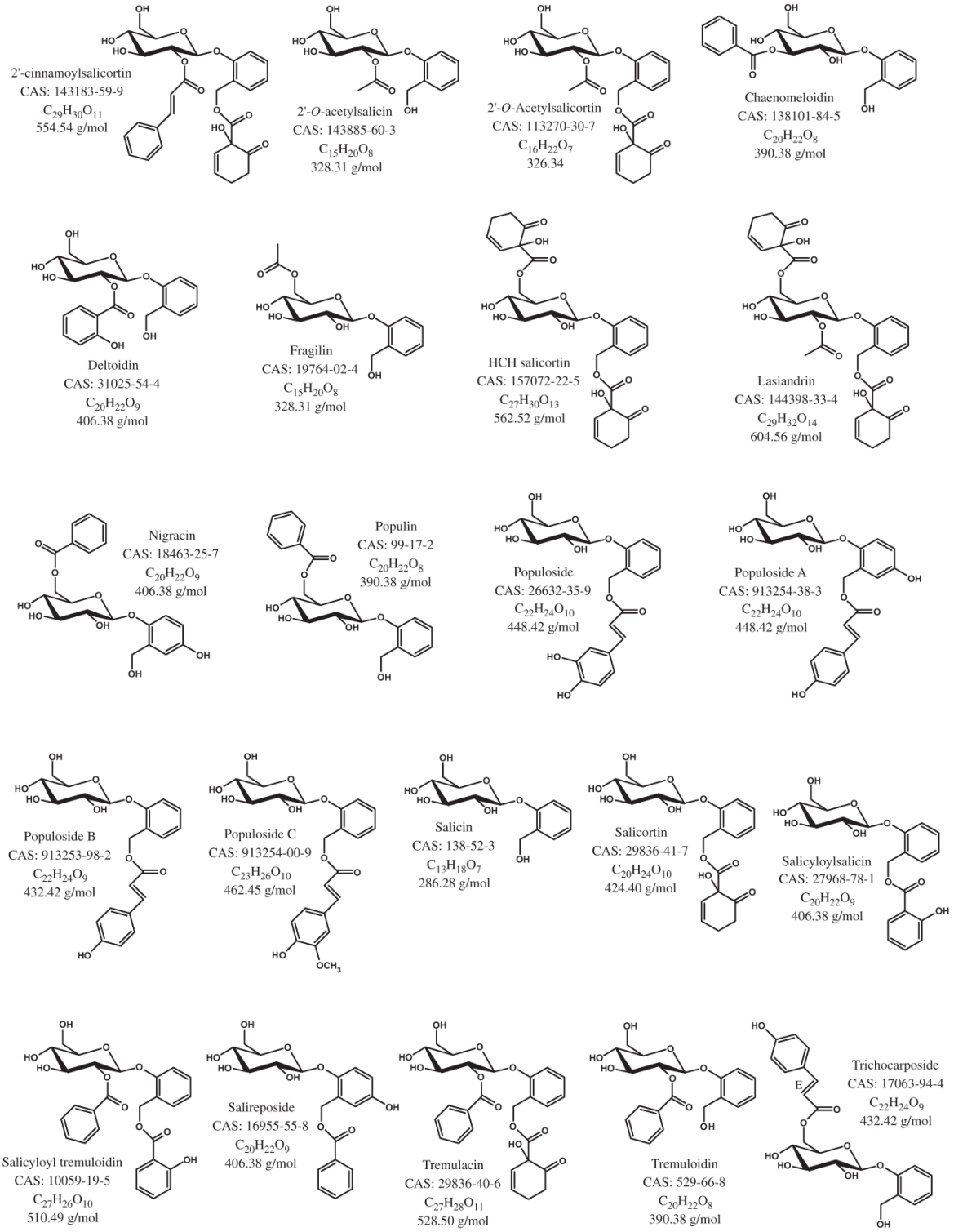

Fig. 1. Names, structures, CAS numbers, molecular formulas and molar weights of salicin-based PGs given in CAS Scifinder listed here in alphabetical order.

PG profiles may even facilitate plant species identification (Julkunen-Tiitto, 1985). Comparisons among and within clones of a single species showed that inter-clonal variation is usually significantly higher than intra-clonal variation, suggesting that PG

Table 1
Phenolic glycosides reported in poplar and willow species. Numbers indicate exemplary references in which the compound has been reported in the tree species listed above.

Compound	Poplar species (*Populus*)														
	P. alba	*P. angustifolia*	*P. balsamifera*	*P. candicans*	*P. davidiana*	*P. deltoides*	*P. fremontii*	*P. gradidentata*	*P. heterophylla*	*P. nigra*	*P. sieboldii*	*P. tremula*	*P. tremuloides*	*P. trichocarpa*	*P. ussuriensis*
2′-Cinnamoylsalicortin															
2′-*O*-acetylsalicin															
2′-*O*-acetylsalicortin															
Chaenomeloidin															
Deltoidin						7, 8									
Fragilin														1	
HCH salicortin						7	2								
Lasiandrin															
Nigracin										1					
Populin								11					11		
Populoside			3		5, 6	7, 8		12							17
Populoside A					5, 6										17
Populoside B					6										
Populoside C					6										
Salicin	1		4	1	6	7, 8				1	14	15, 1	9, 1	1, 4	
Salicortin	1	2	4, 3	1		10, 8	2			1		15, 1	9, 1	1, 4	17
Salicyloylsalicin			3			8								4	
Salicyloyltremuloidin															
Salireposide	1		3	1	6							1	9, 1	1, 4	17
Tremulacin					6	10			13				16, 9	4	
Tremuloidin	1				6							1	9, 1	4	
Trichocarposide			3			8									

Compound	Willow species (*Salix*)																
	S. alba	*S. americana*	*S. cv. aquatica*	*S. arbusculoides*	*S. aurita*	*S. babylonica*	*S. callicarpaea*	*S. calodendron*	*S. caprea*	*S. chaenomeloides*	*S. cinerea*	*S. daphnoides*	*S. decipiens*	*S. elbursensis*	*S. fragilis*	*S.* x *geminata*	*S. incana*
2′-Cinnamoylsalicortin																	
2′-*O*-acetylsalicin																	
2′-*O*-acetylsalicortin																	
Chaenomeloidin										21							
Deltoidin																	
Fragilin	18					18	18	18	15, 1				18		18, 1		18
HCH salicortin																	
Lasiandrin																	
Nigracin																	
Populin							18					18	18		18		18
Populoside																	
Populoside A																	
Populoside B																	
Populoside C																	
Salicin	18, 1	18	15	19	1	20	18	18	15, 1	21	18, 1	18	18	22	18, 1	18	18
Salicortin	18, 1	18	15	19	1	18	18	18	15, 1		18, 1	18	18	22	18, 1	18	18
Salicyloylsalicin																	
Salicyloyltremuloidin										21							
Salireposide																	
Tremulacin										21							
Tremuloidin	18		15			18	18			21					18	18	18
Trichocarposide																	

	S. lapponum	*S. lasiandra*	*S. martiana*	*S. myrsinifolia*	*S. nigricans*	*S. pentandra*	*S. pentandroides*	*S. petiolaris*	*S. phylicifolia*	*S. purpurea*	*S. repens*	*S. schwerinii*	*S. sericea*	*S. triandra*	*S. x rubra*	*S. viminalis*
2′-Cinnamoylsalicortin													28			
2′-*O*-acetylsalicin		23														
2′-*O*-acetylsalicortin		23														
Chaenomeloidin																
Deltoidin														18	18	15, 18
Fragilin	15			15	18	15, 18	25		18	18						
HCH salicortin																
Lasiandrin		23														
Nigracin																
Populin								26		18					18	
Populoside																
Populoside A																
Populoside B																
Populoside C																
Salicin	15		24	15, 1	18	1, 18	25	26	15, 18	18, 1	1	29		18, 1	18	15, 1
Salicortin	15	23		15, 1	18	15, 1			15, 18	18, 1	1		28	18	18	15, 1
Salicyloylsalicin								26		27						
Salicyloyltremuloidin								26		27						
Salireposide								26		1	1			1		
Tremulacin								26						1		
Tremuloidin					18	18		26	15	18				18	18	
Trichocarposide			24													

1, Palo (1984); **2**, Rehill et al. (2005); **3**, Pearl and Darling (1969); **4**, Pearl and Darling (1971b); **5**, Si et al. (2009b); **6**, Zhang et al. (2006); **7**, Picard et al. (1994); **8**, Pearl and Darling (1971c); **9**, Pearl and Darling (1971a); **10**, Clausen et al. (1989); **11**, Pearl et al. (1962); **12**, Erickson et al. (1970); **13**, Pearl and Darling (1977); **14**, Ogawa et al. (2006); **15**, Julkunen-Tiitto (1985); **16**, Thieme and Benecke (1971); **17**, Si et al. (2009a); **18**, Binns et al. (1968); **19**, Evans et al. (1995); **20**, Khatoon et al. (1988); **21**, Mizuno et al. (1991); **22**, Kompantsev and Shinkarenko (1970); **23**, Reichardt et al. (1992); **24**, Fernandes et al. (2009); **25**, Kompantsev and Shinkarenko (1973); **26**, Steele et al. (1972); **27**, Pearl and Darling (1970a); **28**, Nichols-Orians et al. (1992); **29**, Kompantsev and Glyzin (1973).

abundance is primarily genetically determined. Significant variation (up to fivefold) among clones of one species grown under comparable conditions was reported for *Salix myrsinifolia* (Julkunen-Tiitto and Meier, 1992b), *Salix sericea* (Nichols-Orians et al., 1993), *Salix daphnoides*, *Salix pentandra*, and *Salix purpurea* (Förster et al., 2010). *Populus tremuloides* has been subject to extensive investigation in the greenhouse (Lindroth et al., 2001), in common garden experiments (Osier and Lindroth, 2004, 2006) and in the field (Lindroth and Hwang, 1996). In each of these three settings, the genotype was recognized as the key determinant of PG concentration over abiotic factors like nutrient availability, CO_2 concentration and artificial defoliation. However, differences between two clones can also originate from different developmental stages of the investigated plants, as discussed below. The disentanglement of genotypic regulation from other factors is a major problem especially under field conditions.

3.2. Ontogenetic variation

Besides genetic control, PG concentrations in Salicaceae are also strongly dependent on plant ontogeny. The literature on PG content in species of the Salicaceace consistently documents the highest PG levels in juvenile tissue with a steady decrease as plants mature. In *P. tremuloides*, foliar PG concentration exponentially declines with tree age (Donaldson et al., 2006b). Young trees are thus significantly better defended with PGs in a relative sense than mature trees (Erwin et al., 2001), a pattern which has also been observed for juvenile and mature twigs within one tree (Tahvanainen et al., 1985a). Moreover, it was shown that even within a shoot, leaf age has a negative correlation with PG abundance (Bingaman and Hart, 1993; Kleiner et al., 2003). However, 7–14 week-old seedlings of *S. sericea* have a low PG concentration which subsequently increases (Fritz et al., 2001; but see Orians et al., 2010). Both *de novo* synthesis and translocation from other tissues may play an important role in the ontogenetic shifts of PGs. The low concentrations in young plants grown from seeds in contrast to plants propagated from vegetative tissue indicate that PGs may be translocated from older tissues into the new shoots. Salicin has been found in phloem exudates of *Populus deltoides* leaves and young, unfurled leaves had the highest concentration, suggesting a translocation of at least this simplest PG (Gould et al., 2007). Yet there is no unequivocal evidence for PG transport in the Salicaceae.

3.3. Seasonal variation in PG content

PG abundance in bark and leaves is also dictated by the growing season. Although the patterns reported are not completely uniform, bark PG concentrations are generally lowest in August and peak between leaf fall and the next leaf flush (Förster et al., 2010; Thieme and Benecke, 1971). From March to July, the PG content in the bark declines sharply, probably due to translocation to the buds, flowers and leaves, and then undergoes a more moderate decrease until August. The foliar PG dynamics are not as thoroughly investigated as those in the bark, but some studies document a decline in leaf PG concentrations throughout the growing season (Lindroth et al., 1987; Thieme and Benecke, 1971; but see Osier et al., 2000b). This may be a result of leaf maturation (see Section 3.2) rather than a strict seasonal effect. Results from a recent study suggest that PG levels may also vary diurnally, but these findings require further validation (Young et al., 2010a).

3.4. Environmental factors

Intrinsic traits (genotype and ontogeny) are the most important determinants of foliar PG levels, but PG content can also be affected by external biotic and abiotic factors. Significant changes of PG

concentration can be caused by increased atmospheric CO_2, O_3 or SO_2 levels (Holton et al., 2003; Julkunen-Tiitto et al., 1995; Kopper and Lindroth, 2003), competition (Donaldson et al., 2006a), nutrient availability and flood or drought stress (Hale et al., 2005; Lower et al., 2003). Although rarely studied, pathogens may also have an effect on the accumulation of PGs. These external factors may consequently affect the interaction of the Salicaceae with herbivores but this is beyond the scope of this article.

3.5. Metabolic turnover

PGs were once thought to be rapidly turned over in plant tissue following their formation based on extensive diurnal variation in leaf PG content as well as seasonal declines (Clausen et al., 1989; Thieme, 1965). A turnover rate of over 50% in 48 h was measured for PGs formed from isotopically labeled CO_2 in *P. tremuloides* (Kleiner et al., 1999). However, a well-replicated study using micropropagated plantlets of *S. myrsinifolia* in which PG biosynthesis was inhibited with a phenylalanine ammonia-lyase inhibitor, failed to detect any turnover in leaves and only recorded 0.5% turnover per day in stems (Ruuhola and Julkunen-Tiitto, 2000). The possibility of PG translocation among organs complicates the assessment of turnover, and accurate knowledge of PG dynamics in plants may have to await increased understanding of transport.

4. Biosynthesis

Despite the longstanding interest in PGs, there is little precise information on their biosynthetic pathway. No genes or enzymes involved in PG biosynthesis have been identified, although with the availability of the *Populus trichocarpa* genome and other resources for functional genomics, genetics and transformation studies in poplar species (Jansson and Douglas, 2007), rapid progress in this area can be expected.

To date, a number of *in vivo* feeding experiments conducted with isotopically-labeled precursors have outlined a basic framework of PG formation. Early feeding experiments with *S. purpurea* (Zenk, 1967), confirmed much more recently with *Populus nigra* (Babst et al., 2010) indicated that the salicyl moiety of salicin arises from cinnamic acid, consistent with the general origin of salicylates and other C_6–C_1 benzenoids from C_6–C_3 phenylpropanoid precursors. Cinnamic acid can first be *ortho*-hydroxylated to *o*-coumaric acid which then undergoes side-chain shortening. The high incorporation of labeled *o*-coumaric acid into salicin supports this possibility (Zenk, 1967). Alternatively, chain-shortening to a benzyl derivative can proceed first, an idea that gains credence due to the facile incorporation of benzoic acid, benzaldehyde and benzyl alcohol into salicin (Babst et al., 2010; Zenk, 1967).

Subsequent steps require *o*-hydroxylation and glucosylation, but the sequence of these conversions cannot yet be resolved with current information. The results of Zenk (1967) make a strong case for the *o*-hydroxylation of benzaldehyde to salicylaldehyde followed sequentially by glucosylation and reduction, although *o*-hydroxylation could also occur at the level of the alcohol (Babst et al., 2010). From salicylaldehyde, reduction to salicyl alcohol could precede glucosylation, but the conversion of salicyl alcohol in feeding experiments into both salicin and the non-naturally occurring isomer, iso-salicin (Payyavula et al., 2009; Zenk, 1967) speaks against the intermediacy of salicyl alcohol. The biosynthesis of another salicyloid component, the hormone salicylic acid, has been well-studied (Metraux, 2002; Wildermuth et al., 2001), but this substrate is not incorporated into salicin (Zenk, 1967).

The feeding experiments of Babst et al. (2010) have shed light for the first time on the biosynthesis of a more complex PG, the 1-hydroxy-6-oxocyclohex-2-ene-1-carboxylic acid (HCC) ester salicortin. Like the salicyl moiety, the HCC moiety was shown to be of phenylpropanoid origin, and the participation of benzoates, especially benzoyl-CoA and benzaldehyde, in its formation is likely. The role of the benzoates may reflect a complex interconversion of intermediates as has been found in petunia and snapdragon (Long et al., 2009; Orlova et al., 2006) involving both β-oxidative and non-β-oxidative formation of the benzenoid skeleton and the participation of benzylbenzoate, possibly a direct intermediate of salicortin. A curious result was that the simple PG salicin did not seem to be a direct precursor of salicortin (Babst et al., 2010) as might have been expected. These and many other aspects of the PG biosynthetic pathway, including the identity of the responsible genes and enzymes will likely be resolved in the near future given the array of resources available for such research.

5. Phenolic glycosides in plant–herbivore interactions

5.1. PG effects on mammalian herbivores

In their natural habitats, Salicaceae encounter a variety of mammalian herbivores, most of which are generalist feeders that consume the bark and shoots of poplars and willows during the wintertime when other food is scarce. During the winter months, the bark of Salicaceae contains the highest annual PG concentration, as discussed above (Förster et al., 2010; Thieme and Benecke, 1971), suggesting that mammalian herbivores may have selected for this pattern of ontogeny. Early evidence for the protective properties of PGs against mammals came from browsing hares which were observed to reject the juvenile twigs of aspen and willow in preference to mature twigs even though the energy content of juvenile twigs is considerably higher than that of mature twigs (Bryant and Kuropat, 1980). This was attributed to the high secondary metabolite concentration in juvenile tissue which was shown later to consist largely of PGs. Tahvanainen et al. (1985a) demonstrated that the high PG content of juvenile willow twigs was responsible for their unpalatability in feeding trials with mountain hares (*Lepus timidus*). The PG concentration correlated negatively with the percentage of bark removed and the hares rejected oat grain that was treated with a crude PG fraction isolated from experimental willows. Similarly, the field vole (*Microtus agrestis*) lowered its consumption or totally avoided *S. myrsinifolia* bark when the PG concentration was high (Heiska et al., 2007). Comparable patterns were also observed in long term experiments with common brushtail possums (*Trichosurus vulpecula*), porcupines (*Erethizon dorsatum*) and elk (*Cervus elaphus*) feeding on trembling aspen (*P. tremuloides*) (Bailey et al., 2007; Diner et al., 2009; Pass and Foley, 2000). Elk were introduced to Arizona where they browse heavily on native aspen. Since these herbivores prefer to feed on aspen clones whose bark and foliage is low in PG content (Bailey et al., 2007; Wooley et al., 2008), these clones suffer significantly higher mortality. Selection for high PG genotypes in heavily browsed poplar forests may have large ecological consequences for other herbivores and organisms living there (Bailey et al., 2007). In contrast to most other mammalian species, beavers can use Salicaceae as a major food resource throughout the year. Many poplar and willow species preferentially grow in riparian areas, the natural habitat of beavers. Perhaps as a result of this habitat overlap and long-term coexistence of these taxa, the food choice of beavers is unaffected by PG content (Bailey et al., 2004; Basey et al., 1990).

5.2. PG effects on insect herbivores

Numerous studies have demonstrated that PGs strongly effect the performance of both generalist and specialist insect herbivores

(e.g., Glynn et al., 2004; Hemming and Lindroth, 2000; Matsuki and Maclean, 1994; Osier and Lindroth, 2001; Ruuhola et al., 2001). The majority of studies indicate that PG function as feeding deterrents and reduce the fitness of generalist insects (e.g., Lindroth and Peterson, 1988; Lindroth et al., 1988; Tahvanainen et al., 1985b), but can stimulate feeding (e.g., Kolehmainen et al., 1995) and oviposition (Orians et al., 1997; Roininen et al., 1999) of specialists (see also Section 5.3 on "sequestration") and benefit their performance (Matsuki and Maclean, 1994; Rank et al., 1998).

Most of the recent studies testing the effect of PGs on generalist insect herbivores have been performed with quaking aspen (*P. tremuloides*) and either of two lepidopteran species, the gypsy moth (*Lymantria dispar*) or forest tent caterpillar (*Malacosoma disstria*). Caterpillars of *L. dispar* are negatively affected by high levels of phenolic glycosides when feeding on *P. tremuloides*. When caterpillars were reared on foliage from tree genotypes containing high amounts of phenolic glycosides, developmental time was prolonged, pupal weight declined and the number of eggs produced by females was reduced (Osier et al., 2000a). Young and coworkers (2010b) showed in a recent field study on *P. tremuloides* that when the combined concentrations of the PGs salicin and tremulacin were above a threshold level of 27 mg/g dry wt, damage by the specialist leaf mining lepidopteran *Phyllocnistis populiella* was reduced. When 2nd and 4th instar forest tent caterpillars were fed with leaves from 13 aspen clones differing significantly in foliar constituents such as water, nitrogen and secondary metabolites, phenolic glycoside content explained most of the among-clone variation in caterpillar performance (stadium duration, larval growth and consumption rate) (Hwang and Lindroth, 1997). These observations are in contrast to the results from a multitude of studies on specialist chrysomelid beetles where positive effects of phenolic glycosides on host colonization and performance were documented (e.g., Donaldson and Lindroth, 2004; Ikonen, 2002; Rank, 1992; Rowell-Rahier and Pasteels, 1990).

Compared to *P. tremuloides*, fewer studies have looked at PG effects on generalist and specialist insect herbivores in other poplar species and in the genus *Salix*. Matsuki and Maclean (1994) investigated the effects of certain leaf traits in 10 different *Salix* species on the growth rates of five herbivorous insect species differing in host specificity and taxonomy (two specialist and one generalist chrysomelid beetle, one specialist sawfly and one generalist lepidopteran). Four out of the 10 *Salix* species contained phenolic glycosides in their leaves. The growth rates of the generalist insect herbivores in this study were all affected negatively by the presence of PGs in *Salix*, whereas one of the tested specialists, the willow sawfly *Nematus calais*, performed better on PG-containing trees. The second specialist in this study, *Chrysomela falsa*, was not affected by PGs.

The effects of individual phenolic glycosides on herbivore performance cannot be easily disentangled from other host-plant characteristics such as nutrients and other secondary metabolites (e.g., condensed tannins). Therefore experiments with artificial diets are helpful to understand the role of specific PGs for antiherbivore defense. These studies employed salicin, salicortin and tremulacin, the most abundant PGs in both the genus *Populus* and *Salix* (Lindroth et al., 1987; Palo, 1984). Compounds were incorporated into artificial diet or added to surfaces of leaves with low PG levels (Hemming and Lindroth, 1995; Kelly and Curry, 1991; Orians et al., 1997). When 4th instar *L. dispar* and *M. disstria* caterpillars were fed with aspen leaves supplemented with the PG tremulacin, developmental time of the larvae increased and growth rates decreased (Hemming and Lindroth, 1995). Salicin and salicortin applied to the leaves of *Salix viminalis*, a tree species naturally low in PG levels, had negative effects on larval growth and larval development of the willow beetle *Phratora vulgatissima*, and furthermore adult beetle feeding was diminished when the leaves were supplemented with phenolic glycosides (Kelly and Curry, 1991). In contrast, the specialist leaf beetle *Chrysomela knabi* preferred leaves painted with different salicortin concentrations over control leaves painted with just solvent (Orians et al., 1997).

Most studies investigating the effects of PGs on insect herbivores have been restricted to leaf chewers. Only rarely has the effect of PGs been investigated with respect to sucking insects (see Gould et al., 2007; Zucker, 1982). Furthermore, the studies on leaf chewers have mostly focussed on lepidopterans as generalists and chrysomelid beetles as specialists. Future research should attempt to redress these imbalances with respect to dietary specialization, taxonomy and feeding guilds to determine if PGs act as plant defense compounds against a broad spectrum of insect herbivores. A further shortcoming in the existing literature is the narrow range of insect developmental stages that has been tested, mostly only a few juvenile instars and/or adults (but see Osier et al., 2000a). Furthermore, the effects of PGs on the most important fitness parameter of a species – reproductive success – have not received much attention (but see Lindroth et al., 1997).

5.3. PG sequestration by herbivores

Like other defensive secondary metabolites, PGs have been reported to be utilized by specialized herbivores species for their own defense, such as by the viceroy butterfly (*Limenitis archippus*, Nymphalidae) and larvae of the coleopterans, *Chrysomela populi* and *Phratora vitellinae* (Prudic et al., 2007; Rowell-Rahier and Pasteels, 1990). These specialized leaf beetles (Chrysomelidae) are capable of converting salicin of plant origin to salicylaldehyde which is stored in glandular reservoirs and secreted upon attack by natural enemies. The plant origin of salicin was proven by Pasteels et al. (1983) in experiments with ^{14}C-labeled salicin. Mechanistic studies on the uptake of thiosalicin, the synthetic S-glucoside analog of salicin, in *C. populi* and *P. vitellinae* indicate that intact salicin is transported to the defensive glands without prior deglucosylation (Kuhn et al., 2004). However, the gland secretions exhibit a very high glucosidase and oxidase activity, suggesting that salicin is cleaved *in situ* and the liberated salicyl alcohol is oxidized to salicylaldehyde (Pasteels et al., 1990). The latter compound deters predators like ants and also has antimicrobial properties (Opitz and Müller, 2009) (see also Section 5.5). Females of some PG-sequestering species are able to incorporate salicin into their eggs. When neonate larvae hatch from these eggs, they contain only salicylaldehyde but no salicin, which indicates that salicin has been converted to salicylaldehyde right before eclosion (Pasteels et al., 1986). Both compounds protect the egg and neonate larva from predators like ants during a very vulnerable developmental stage. It is also notable that apart from the predator deterring properties of salicylaldehyde, the sequestration of salicin provides a substantial amount of glucose. The energy recovery from the glucose balances or may even exceed the metabolic costs of sequestration (Kearsley and Whitham, 1992). Since adult weights were equal or even higher when chrysomelid larvae were provoked to secret their entire gland contents daily, compared to larvae that were unmolested, this suggests that continuous sequestration has no metabolic costs and may even be beneficial. Rowell-Rahier and Pasteels (1986) observed that *P. vitellinae* larvae can cover up to 30% of their energy demand with the glucose that is cleaved from salicin, when larvae were forced to secrete their gland contents at 24 h intervals. Assuming that detoxification requires fewer resources than sequestration, other non-sequestering herbivores may also utilize PGs as an energy source if they are able to detoxify the noxious aglycones efficiently. While sequestration of salicin by specialized beetles is well understood, this compound is not very abundant (relative to other PGs) in many poplars and willows.

More research is needed on whether sequestering species can also exploit more complex PGs and how they cope with their toxic components *vide infra*. Since host plant PG abundance and composition affects the food choice of specialist beetles, the costs and benefits of sequestering different PGs may not be equivalent.

5.4. PG induction

The allocation of resources to plant defense may be costly if these resources are no longer available for investment in plant growth and reproduction. Thus the production of certain defense compounds only upon attack by herbivores is considered a cost-saving strategy (Zangerl, 2003). Although phenolic glycosides are constitutively present in Salicaceae species, they are also inducible upon herbivore attack or artificial defoliation. However, the examples from the literature do not present a clear pattern of induction. Clausen et al. (1989) were the first to show short-term induction of the phenolic glycosides salicortin and tremulacin in quaking aspen (*P. tremuloides*) 24 h after simulated large aspen tortix herbivory. In an experiment by Stevens and Lindroth (2005), quaking aspen trees showed PG induction 8 weeks after excessive defoliation by both, artificial damage and forest tent caterpillar feeding. The induction occurred in undamaged leaves that were produced subsequent to the defoliation. The authors term this phenomenon "intermediate-delayed induced resistance" (IDIR) and argue that this is a defense strategy that may protect trees efficiently against late season herbivory and damage to compensatory growth (Stevens and Lindroth, 2005). When young quaking aspen was attacked by the epidermal leaf mining species *Phyllocnistis populiellea*, both salicortin and tremulacin were induced but only 9–12 days after leaf mining started. By that time, damage levels were greater than 5% of the leaf surface area. It is conceivable that quaking aspen only induces PG production after a certain damage threshold has been reached (Young et al., 2010a). Fields and Orians (2006) observed an "all-or-none" response of PG induction when they investigated the effect of three different types of herbivore damage (two species of beetles, with one represented as adults and as larvae) on *S. sericea*. In this study, induction of 2-cinnamoylsalicortin occurred only in young systemic leaves four days after the actual damage. Interestingly, *Plagiodera versicolora* larvae caused a decrease of salicortin in local damaged leaves. Both the adults of this beetle species and the adults of *Calligrapha multipunctata* caused no change in salicortin levels of *S. sericea*.

The examples above show clearly that the timing, intensity and location (local vs. systemic) of phenolic glycoside induction in Salicaceae depends on a variety of factors such as the identity of the tree species, the tree genotype (Julkunen-Tiitto et al., 1995; Ruuhola et al., 2001), and the attacker, including its developmental stage and feeding strategy. However, further experiments are needed to investigate whether the phenolic glycosides are really *de novo* synthesised (as suggested by Ruuhola et al., 2001) or just translocated (as suggested by Clausen et al., 1989), as this will certainly affect resource allocation and energy costs for the tree. PG induction patterns may also differ depending on ontogeny. Given that young trees have especially high constitutive levels of PGs, a further increase in PG contents may not provide a gain in protection against insect herbivores as it could in older trees, where PG levels are low to begin with. Therefore, one might predict that older trees with low constitutive PG levels might effectively defend themselves against generalist herbivore attack through PG induction.

5.5. PG effects in higher trophic-interactions

There are a few examples in the literature demonstrating that after ingestion by herbivores PGs also play an important role in higher order trophic interactions. However, whether these interactions benefit the plant itself is not straightforward. Similar to the effects of plant PGs on generalist insect herbivores, these compounds are defenses against generalist insect predators after sequestration by specialist chrysomelid beetles. Either as intact PGs or in the form of PG-derived salicylaldehyde they deter generalist insect predators, including ants (Kearsley and Whitham, 1992; Matsuda and Sugawara, 1980; Pasteels et al., 1983), ladybird beetles (Cha et al., 2009; Denno et al., 1990), mantids (Cha et al., 2009), spiders (Palokangas and Neuvonen, 1992) and even birds (Müller et al., 2006). Therefore specialist insect herbivores can benefit from feeding on trees with high phenolic glycoside contents (Orians et al., 1997) not only because of the energetic value of these chemicals (in form of sugars) but also due to the fact that these compounds themselves and the sequestration product (salicylaldehyde) act as defense compounds against predators and parasitoids. Pasteels et al. (1986) reported that the amount of salicin in a single *Chrysomela* egg is toxic enough to kill an individual ant. Thus specialized beetles are able to use plant derived phenolic glycosides to effectively defend themselves against generalist predators in more than one stage of their ontogeny.

Even generalist insect herbivores that are negatively affected by PGs themselves can benefit from the deterrent effect of these compounds on higher trophic levels. Black-capped chickadees, generalist predators, preferred to feed on generalist *L. dispar* caterpillars that were fed leaves with low levels of phenolic glycosides as compared to caterpillars reared on leaves rich in these compounds (Müller et al., 2006).

For specialist insects at higher trophic levels, PGs may have either positive or negative effects. *Cotesia melanoscela*, a gypsy moth parasitoid, was shown to have lower cocoon weights and higher mortality when its host was reared on artificial diet containing PG compared to a control diet (Roth et al., 1997). In contrast, *Megaselia opacicornis*, a specialist parasitoid of the poplar feeding coleopteran, *Chrysomela lapponica*, uses the defensive secretions of its host (mostly salicylaldehyde) to locate it (Zvereva and Rank, 2004). In a field experiment where beetle larvae secretions were regularly removed starting with third instar larvae 6 days before pupation, oviposition by these parasitoids was reduced 7.5-fold as compared to untreated control larvae (Zvereva and Rank, 2004). This example shows nicely that specialized insect herbivores cannot always escape predation or parasitism by sequestering plant derived toxins, as there may also be specialized enemies that can overcome these defenses and use them for their own benefit.

5.6. PG effects on herbivore–pathogen interactions

As part of their function in direct defense, PGs can amplify the negative effect of pathogens on herbivores. Cook et al. (2003) studied the combined effects of Gypchek, a commercial gypsy moth nuclear polyhedrosis virus preparation, and the PG salicin on the survival of *L. dispar*. When second instar caterpillars fed on a formulation containing Gypchek enriched with salicin, caterpillar mortality was significantly higher as compared to the treatment where only Gypchek was present in the formulation. This result indicates that the phenolic glycoside salicin boosts anti-viral activity. When phenolic glycosides (salicortin and tremulacin) were incorporated in artificial diet containing the bacterium *Bacillus thuringiensis*, the effects on *L. dispar* caterpillar mortality were also increased (Arteel and Lindroth, 1992).

Given the way PGs not only affect insect herbivores but also shape their interaction with higher trophic levels, including predators and parasitoids, these compounds have the potential to impact the composition of whole arthropod communities found on species of Salicaceae (e.g., Hwang and Lindroth, 1997; Rowell-Rahier, 1984; Soetens et al., 1991; Wimp et al., 2007).

6. Herbivore processing of PGs and mode of action

6.1. Mammals

Mammalian herbivores typically process plant defenses such as PGs in their kidneys and liver. For example, common brushtail possums (*T. vulpecula*), Australian marsupials, that were fed salicin-supplemented food were reported to eliminate more than half of the ingested salicin in the urine, primarily in the form of this glycoside as well as the water soluble salicyl alcohol glucuronide (Fig. 2) (McLean et al., 2001). Although this transformation is not a simple oxidation of salicin, but requires its cleavage and reconjugation of the aglycone with glucuronic acid, the metabolic costs are considered to be low (McLean et al., 2001). Pass and Foley (2000) demonstrated that common brushtail possums limit their food uptake in no choice experiments so that they do not consume more than a threshold dose of salicin per day, but negative post-digestive consequences of salicin intake were not evident. Therefore, it is likely that the deterrent effect of salicin on possums is pre-digestive. This may also be true for mountain hares as well which are deterred by the smell of volatile 6-HCH that originates from salicortin in juvenile poplar twigs (Clausen et al., 2010; Reichardt et al., 1990).

6.2. Insects

Despite numerous studies about the effects of PGs against insects, no definitive information on their metabolism is available, even for simple PGs which seem to be efficiently processed. Although PGs containing HCC, such as salicortin and tremulacin, exhibit a high biological activity against insect herbivores, simple PGs like salicin are much less active. The polyphagous *Spodoptera eridania*, *Papilio glaucus glaucus* and the aspen adapted subspecies *Papilio glaucus canadensis* are practically unaffected by salicin (Lindroth and Peterson, 1988; Lindroth et al., 1988). In contrast, salicortin and especially tremulacin significantly prolonged the duration of the penultimate instar of larval *S. eridania* and *P. g. glaucus* when applied to the leaves of accepted food plants in natural concentrations (Lindroth and Peterson, 1988; Lindroth et al., 1988). Slower development was a result of reduced consumption and decreased conversion of food, most likely caused by mid-gut lesions. Possible metabolic reactions of PGs have been postulated mainly on the basis of *in vitro* approaches, such as investigation of the enzyme activity of dissected caterpillar guts after PG digestion and chemical degradation under specified conditions, and the results are summarized in Fig. 2.

PGs exhibit two obvious attack sites for enzymatic breakdown. The glycosidic bond between the sugar and the salicyl alcohol moiety, is a potential target for β-glucosidases, while the ester bonds between the salicin core structure and organic acid moieties in complex PGs are targets for esterases. Interestingly, β-glucosidase and esterase activity showed an inverse behavior upon PG ingestion in some studies. β-glucosidase activity is down-regulated in *L. dispar* caterpillars upon PG feeding, indicating that the catalyzed reaction may have toxic consequences (Lindroth and Bloomer, 1991; Lindroth and Hemming, 1990; Lindroth et al., 1988). This is also supported by the fact that aspen adapted *P. g. canadensis* exhibits a lower mid-gut β-glucosidase activity than the close relative *P. g. glaucus*, which does not feed on Salicaceae and is susceptible to PGs (Lindroth, 1988). How might β-glucosidase-catalyzed PG decomposition lead to toxicity if the products are a carbohydrate or carbohydrate ester and salicyl alcohol or a salicyl alcohol ester? One possibility is that the salicyl alcohol ester released spontaneously decomposes to form a toxic product (Fig. 2). Clausen et al. (1990) reported hydrolysis of salicortin by β-glucosidases and the formation of 6-hydroxy-6-(2-hydroxybenzyl)cyclohex-2-en-1-one ([**3**] in Fig. 2) which is considered toxic. The authors observed the spontaneous decarboxylation of the salicyl alcohol ester aglycone remaining after glucosidase action by respirometry and detected product **3** by GC/MS. **3** was proposed to be the recombination product of the putative intermediates 6-methylidenecyclohexa-2,4-dien-1-one ([**1**] in Fig. 2) and cyclohexa-1,3-diene-1,2-diol ([**2**] in Fig. 2), that are formed during the decarboxylation. During the assay, the β-glucosidase activity decreased irreversibly, which was suggested to be due to reactions of **3** with the enzyme at or near the active site (Zhu et al., 1998). Julkunen-Tiitto and Meier (1992a) also demonstrated that a purified β-glucosidase from almonds decomposes salicin and salicortin. However, esterification of the carbohydrate, as occurring in tremulacin, prevented the hydrolysis. In contradiction to the findings of Clausen et al., Julkunen-Tiitto and Meier detected salicyl alcohol and 6-hydroxycyclohexen-2-one (6-HCH) when they administered salicortin to the almond β-glucosidase and found no traces of compound **3**. An alternative reaction mechanism was proposed where 6-HCH tautomerizes from **2** and salicyl alcohol forms from **1** after the addition of water. With regard to the toxicity of PGs, the formation of 6-HCH may be of great relevance, as it can react to form toxic catechol (Clausen et al., 1989). This conversion under basic and aerobic conditions was reported much earlier (Pearl and Darling, 1970b) and is especially notable since catechol has been proposed as substrate for polyphenol oxidases (PPO) (Haruta et al., 2001) (Fig. 2). PPOs are wound inducible enzymes in *P. tremuloides* and catalyze the reaction of catechol to the highly reactive *o*-quinone. Perhaps they participate in the bio-activation of PG metabolites to toxic products. Similar to 6-HCH, *o*-quinone is a potent electrophile capable of reacting with the nucleophilic groups of biomolecules (e.g., S–H or $-NH_2$) like proteins (Fig. 2) (Clausen et al., 1989; Haruta et al., 2001).

In contrast to β-glucosidases, esterases have been shown to be up-regulated upon PG ingestion in *P. g. canadensis*, *L. dispar* and *M. disstria* (Hemming and Lindroth, 2000; Lindroth, 1991). Lindroth et al. (1988) speculated that esterases are responsible for the enzymatic cleavage of the salicyl alcohol HCH ester (Fig. 2), thus initiating an unknown detoxification mechanism of the toxic HCH moiety. In fact, it was shown, that supplementation of PG containing food with an esterase inhibitor significantly increases mortality and reduces performance of *M. disstria* (Lindroth and Bloomer, 1991). The hypothesis also provides an explanation for the stronger toxicity of tremulacin compared to salicortin at an equimolar concentration: the benzoyl ester moiety of tremulacin is an alternative substrate for esterases and may serve as a competitive esterase inhibitor (Lindroth et al., 1988). This assumption could not be proved by Julkunen-Tiitto and Meier (1992a), who investigated the *in vitro* decomposition of tremulacin by esterases and found that the salicyl alcohol HCC ester is easily cleaved, whereas the resulting tremuloidin was relatively resistant to ester hydrolysis. Additionally, they found that the toxic metabolites, 6-HCH and catechol, were formed after salicortin and tremulacin were subjected to esterase activity. However, it was recognized that the artificial *in vitro* conditions of the assay may not match the *in vivo* situation.

To date, the involvement of β-glucosidases and esterases in the metabolism of PGs has not yet been definitely proven. The β-glucosidase assays mentioned above may not accurately reflect *in vivo* conditions since they have been performed at a slightly acidic pH value that does not match the alkaline mid-gut pH of most lepidopteran larvae (Johnson and Felton, 1996). Moreover, it remains unclear, if the enzymes that initiate the PG breakdown originate from the herbivore or come from the ingested plant. In the latter case, the formation of toxic metabolites is inevitable and herbivores would need mechanisms to deactivate the plant enzymes or an efficient detoxification system to cope with the

toxic molecules. Early experiments by Mattes et al. (1987) demonstrated the formation of 6-HCH when salicortin was incubated with an enzyme preparation from winter dormant *Populus balsamifera* internodes. Incubation with a boiled enzyme preparation

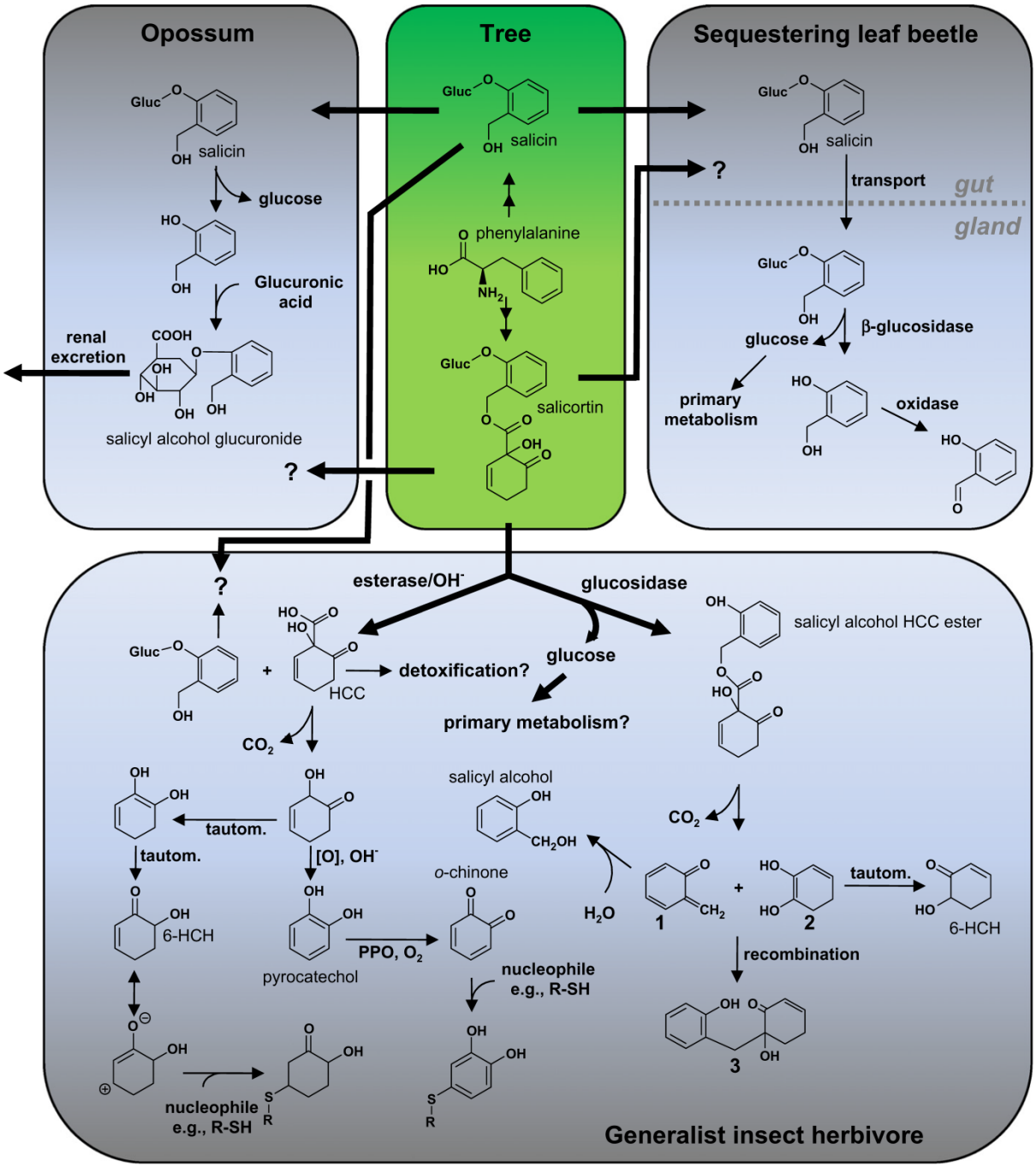

Fig. 2. Proposed metabolic breakdown of salicin and salicortin in possums, sequestering leaf beetle larvae and generalist caterpillars. In opossum the glycosidic bond of salicin is cleaved. The resulting salicyl alcohol is reconjugated with glucuronic acid and excreted. Sequestering herbivores transport intact salicin to special glands were it is transformed to salicylaldehyde, a defense compound released upon attack by predators. The glucose is processed in primary metabolism. The metabolism of complex PGs like salicortin is unknown in opossum or sequestrating insects. In generalist herbivores two metabolic pathways of salicortin are conceivable. Alkaline or enzymatic cleavage liberates 1-hydroxy-6-oxocyclohex-2-ene-1-carboxylic acid (HCC) which decarboxylates to form the electrophiles, 6-hydroxycyclohex-2-en-1-one (6-HCH) or *o*-quinone through polyphenol oxidase (PPO) catalysis. Glucosidase activity liberates the salicyl alcohol HCC ester from salicortin which reacts to form 6-methylidenecyclohexa-2,4-dien-1-one (**1**) and cyclohexa-1,3-diene-1,2-diol (**2**). **1** and **2** form toxic 6-hydroxy-6-(2-hydroxybenzyl)cyclohex-2-en-1-one (**3**). Alternatively, **1** can react with water to form salicyl alcohol and **2** can tautomerize to 6-HCH. The metabolic fate of salicin in generalist insect herbivores has not yet been elucidated.

did not lead to formation of 6-HCH, suggesting an enzyme catalyzed process. In fact, intact *P. balsamifera* was recently reported to produce 6-HCH, apparently from salicortin, during the winter months (Clausen et al., 2010). However, enzymes may not be at all involved in the bio-activation of PGs. When salicortin was exposed to an alkaline buffer (pH 9) with a similar pH to the mid-gut of *Operophtera brumata* (pH 9.2–9.5), it spontaneously decomposed to form 6-HCH and catechol (Ruuhola et al., 2003). Irrespective of the mechanism, complex PGs such as salicortin have been shown to be completely degraded during digestion in the generalist *O. brumata* (Ruuhola et al., 2001). The frass of this PG-sensitive species contained mainly salicin as a breakdown product.

In summary, the metabolism of certain complex ester-containing PGs in insects apparently results in the formation of reactive molecules, either 6-HCH or *o*-quinones, which are likely to combine with other substances in the insect gut. This may harm insects directly due to reactions with the gut wall or peritrophic membrane. Alternatively, reactive derivatives of PGs may inactivate digestive enzymes or nutrients themselves, resulting in impaired nutrition. Possible detoxification or other mechanisms for resistance to PGs in insect herbivores have not yet been explored. Moreover, the metabolic fate of substituents other than HCC remains unclear. Further investigations on PG metabolism in insect herbivores are needed to understand the molecular bases of their toxicity to some herbivores and lack of effect on others.

7. Conclusion, perspectives and suggestions for further research

Numerous studies in recent years have provided evidence for the fundamental role of phenolic glycosides in interactions of plant species of the Salicaceae family with their natural herbivore enemies. With respect to their foliar biomass proportion, PGs are amongst the most abundant known secondary metabolites in Salicaceae. However, most studies on the role of PGs in plant–herbivore interactions have been rather descriptive and mechanistic knowledge of their biotic interactions is scarce. Future research to elucidate the biosynthesis of phenolic glycosides in plants should provide a range of molecular and genetic tools to better characterize their roles in plant defense and mode of action. In addition, it may be useful to redefine the terminology used for referring to these substances. Though frequently used, "phenolic glycosides" and "salicylates" are not completely accurate for several reasons. PGs can be applied to a vast number of secondary metabolites with little chemical similarity to salicin. Moreover, salicin derivatives should not even be referred to as phenolics, if this term is used only for molecules that actually exhibit free phenol groups. We also do not recommend the name salicylates since it does not appear that salicylic acid is an intermediate in their biosynthesis. Additionally, the term could lead to chemical misunderstandings since it is already used for the salts and esters of salicylic acid, which are important plant hormones. We thus suggest the term "salicinoids" as a more precise way to describe the diverse array of salicin-derived products found in the Salicaceae.

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3.3. Manuscript III: Metabolism of salicinoids in the gypsy moth (*Lymantria dispar)*

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Abstract

Salicinoids are low molecular weight phenolics produced by species in the family of the Salicaceae and contribute considerably to the direct defense of these plants against herbivores. Generalist herbivores especially suffer from a reduced performance when feeding on diets containing these compounds. All salicinoids are composed of salicin esterified to a variable number of organic acids and the presence of some ester functions was shown to influence their bioactivity. Possible routes of herbivore salicinoid metabolism have been proposed but are not yet validated. Here, we systemically investigated the *in vivo* processing of salicinoids in the gypsy moth (*Lymantria dispar*). In a first experiment we fed caterpillars sequentially diets supplemented with three salicinoids (salicin, salicortin and tremulacin) and screened the feces for metabolites. The modular salicinoid composition permitted an unequivocal assignment of metabolites to specific building blocks. All tested salicinoids were broken down to salicin and a previously unknown salicin phosphate, which were concluded to be a metabolite of the salicin core structure occurring in all salicinoids. Salicortin and tremulacin were found to be converted to catechol glycoside and catechol glycoside phosphate in the digestive system. These metabolites originate from the HCC moiety that both compounds share in contrast to salicin. Additionally, *L. dispar* converted tremulacin into hippuric acid, a widespread metabolite of benzoic acid, which is part of tremulacin but not of salicin or salicortin. In a second experiment we reared *L. dispar* on different diets supplemented or devoid of salicinoid components and recorded the weight gain for 15 days. Thereafter, these caterpillars were fed with leaf discs of *Populus tremula* x *tremuloides* and metabolite excretion rates in the context of the diet pre-treatment were investigated. Excretion rates of metabolites showed that the formation of the two so far unrecognized phosphorylated conjugates is affected by previous nutrition.

Introduction

The interaction between plants and their herbivores reaches back several million years and is one of the longest relationships between higher organisms on our planet (Labandeira 2007). Although ostensibly defenseless, plants face their attackers with a hidden arsenal of sophisticated countermeasures. One of the simplest is the production of harmful secondary metabolites with adverse effects on herbivores upon ingestion. This strategy, called direct defense, is supposedly omnipresent in the plant kingdom. It is also a widespread phenomenon in plants to store these toxic compounds in an inactive form in order to avoid auto-toxicity (Pentzold *et al.* 2013). These inactive forms can be glycosides stored separately from the corresponding glucosidases that initiate bioactivation by cleaving the glycosidic bond enzymatically (Pentzold *et al.* 2013). The released aglycone typically undergoes a further, often spontaneous chemical conversion to form the actual toxin, which negatively impacts the metabolic processes in the herbivore to reduce fitness or increase mortality. The beginning of this reaction cascade and the physiological impact on the herbivore are well documented for an array of secondary metabolites. Well known examples are the glucosinolates (Halkier & Gershenzon 2006), iridoid glycosides (Dobler, Petschenka & Pankoke 2011) or benzoxazinoids (Dixon *et al.* 2012), which all react to putatively toxic electrophiles. The *in vivo* existences of electrophiles, and their metabolic targets, are difficult to evaluate due to a marked and unspecific reactivity and thus the further metabolic fate is often unknown.

As a reaction to the toxins produced by their host plants herbivores evolved mechanisms to decrease or avoid toxicity and can even sequester the secondary metabolites to use them for their own defense. Sequestration and sophisticated avoidance require adapted metabolic machineries and are typically restricted to specialist herbivores that feed on a limited number of taxonomically related plant species with similar secondary chemistry (Opitz & Müller 2009). Herbivores covering a large range of host species (i.e. generalists *sensu* Schoonhoven *et al.*) encounter all sorts of plant defense compounds and cope with them by simple avoidance and detoxification strategies. Detoxification of xenobiotics is a ubiquitous process in all living organisms and typically comprises chemical modification (phase I), conjugation (phase II) and excretion (phase III) of foreign compounds. Chemical modification often averts immediate toxicity and makes the molecule suitable for conjugation, which increases water-solubility and facilitates transport processes necessary for excretion. In insects, these mechanisms are not well understood and our knowledge is restricted to single detoxification steps of few defense compounds in a small number of herbivore species (reviewed in Després, David & Gallet 2007).

Salicinoids are very appropriate for metabolic studies due to their well-known anti-herbivore activity, high abundance in poplars and willows and the previous work on their reactivity. The impact of salicinoids on the performance of herbivores has been subject of studies regarding various insect species, including *Papilio glaucus* (Lindroth 1988b), *Choristoneura conflictana* (Clausen *et al.* 1989), *Malocosoma disstria* and *Lymantria dispar* (Hemming & Lindroth 1995) or *Operiphtera brumata* (Ruuhola, Tikkanen & Tahvanainen 2001). Yet their metabolism in insect herbivores is only poorly understood (Boeckler *et al.* 2011). Depending on parameters, such as plant species and age, herbivorous insects encounter salicinoid concentrations of more than 1 % dry weight and it has been observed that more complex salicinoids are quantitatively degraded (Lindroth, Scriber & Hsia 1988; Ruuhola, Tikkanen & Tahvanainen 2001). Therefore, at least the major fecal metabolites should be detectable. Salicinoid degradation has been previously observed in *in vitro* decomposition assays with commercial enzymes (Clausen, Keller & Reichardt 1990; Julkunentiitto & Meier 1992) or at a specific pH (Ruuhola, Julkunen-Tiitto & Vainiotalo 2003), and in studies of metabolic conversions in bacteria (Zhu *et al.* 1998; Sonowal *et al.* 2013), sequestering leaf beetles (Kuhn *et al.* 2004), possum (McLean *et al.* 2001), rats and humans (Knuth *et al.* 2013). However, empirical evidence of salicinoid metabolism in generalist herbivores is still lacking, although these compounds are assumed to significantly impact the arthropod community of poplars and willows (Boeckler, Gershenzon & Unsicker 2011).

In the present study we aimed to identify *in vivo* salicinoid metabolites in caterpillars of the generalist herbivore *Lymantria dispar*. In a second approach we aimed to elucidate how effectively ingested salicinoids are converted into these metabolites and how this process is influenced by a continuous ingestion of putatively harmful plant metabolites.

Material and Methods

Identification experiment

In order to identify putative salicinoid metabolites, the feces extracts of gypsy moth caterpillars reared on an artificial diet (that contains no salicinoids) were compared to the feces extracts of individuals reared on the same diet supplemented with 10 % dry weight (DW) salicin (Sigma), 2.5 % DW salicortin or 2.5 % DW tremulacin (kindly provided by Richard Lindroth and Bernd Schneider). The artificial diet was prepared from commercial gypsy moth diet (MP Biomedicals, Illkirch, France) which was thoroughly mixed with hot water (approx. 80 °C) in a 1:4 weight:weight ratio. The diet slurry was allowed to cool down and offered to the 4-5th

instar caterpillars. When the diet was supplemented with salicinoids, these were added to the hot water right before the mixing process. A preliminary experiment had shown that tremulacin, the salicinoid most susceptible to degradation, is stable when heated for 15 min to 90 °C in an aqueous solution. Degradation or reactions with diet constituents could still not be excluded and candidate metabolites were only considered if they were also found in the feces extracts of larvae fed with poplar leaves. Each diet treatment contained of at least two replicates and in each replicate at least 250 mg (FW) diet were offered to 4-7 caterpillars (depending on their size) starved for 24 h. Caterpillars typically consumed the entire diet within a few hours and the feces excreted during the following 48 h was collected and freeze-dried. Dry feces samples were extracted with 300 µL MeOH, solid debris was spun down and the supernatant was analyzed by LC/MS as described below.

Conversion experiment

After the identification of several metabolites, we investigated 1) which proportion of ingested salicinoids is excreted in the form of the compounds identified and 2) if the conversion differs when the caterpillars are adapted to specific salicinoid components over a longer period of time. In order to "adapt" the digestion, we reared 2 week old caterpillars artificial diet supplemented with salicin (SAL), catechol (CAT), benzoic acid (BEN), a mixture of the three compounds (MIX), poplar leaves (POP) and a control diet (CON). The adaptation phase was simultaneously used as performance study and the experimental details are described below. After the adaptation 10 caterpillars of every treatment were introduced in a no-choice scenario with *P. tremula x tremuloides* leaf discs to determine metabolic conversion rates. In detail, we calculated excretion rates, i.e. percentage of ingested material recovered in the feces (100* mol feces/mol ingested). Such calculations required an exact knowledge of the amount eaten in addition to the concentrations of salicinoids or their metabolites in the diet and feces. *P. tremula x tremuloides* produces few low molecular weight phenolics except for salicortin and tremulacin (greater amounts) and salicin and tremuloidin (smaller amounts) (Boeckler *et al.* 2014) and it was assumed that the leaves contained no other significant sources of salicyl alcohol, HCC or benzoic acid. To determine all diet parameters, a manual hole puncher was used to punch discrete discs (30 mm) from the lamina (midveins were avoided) of leaves from trees grown in a greenhouse (tree height approx. 1.5 m). Depending on size, each leaf provided four or six discs that were assumed to be homogeneous in water and salicinoid content. One reference disc per leaf was not offered to the caterpillars but weighed (FW), flash-frozen in liquid nitrogen and freeze-dried before weighing (DW), grinding and chemical analysis as

described below. All other discs of the leaf were weighed (FW) and offered to an individual caterpillar in a 90 mm petri dish lined with a Whatman filter paper (diameter 90 mm) moistened with 750 µL water. The filter paper covered the ceiling of the petri dish to avoid contact with the excreted feces. The caterpillars were allowed to feed for 18 h before the leaf disc was removed, flash frozen, freeze-dried, weighed (DW), ground and analyzed. The post-experimental leaf disc dry weight was subtracted from the initial dry weight (calculated on the basis of the FW by aid of the water content of the reference disc of the same leaf) to give the ingested leaf mass (DW). After the 18 h feeding period, the caterpillars were left in the petri dish for 24 h without diet to ensure quantitative excretion of feces. Then the feces were collected, frozen at -20 °C, freeze-dried, weighed (DW) and analyzed (described below). Across all treatments some caterpillars excreted more feces than leaf material was ingested. This may be due to a low precision of the method applied but could also have biological reasons, for example an inappropriate diet. Such data points were excluded together with all data where the caterpillars showed no activity during the 18 h feeding period. The resulting number of replicates was as follows: CON:5; SAL:6; BEN:6, CAT:8; MIX:6 and POP:8.

Performance experiment

We reared two week old caterpillars on five different diets that contained at least one putative salicinoid metabolite and a control diet. The former consisted of *Populus tremula* x *tremuloides* leaves (POP) and artificial diets supplemented with salicin (SAL), benzoic acid (BEN), catechol (CAT) and a mixture (MIX) of all three compounds in concentrations typically occurring in *P. tremula* x *tremuloides* leaves (200 µmol/g salicin, 150 µmol/g catechol and 50 µmol/g benzoic acid) on a dry weight basis as found in Boeckler *et al.* (2014). Catechol was offered instead of the 1-hydroxy-6-oxocyclohex-2-ene-1carboxylic acid (HCC), which is not commercially available and is likely to form catechol in the caterpillar gut based on our previous results and reports by Ruuhola, Tikkanen and Tahvanainen (2001). The catechol concentration was selected assuming a quantitative conversion of HCC. As artificial diet, we employed the same wheat germ diet used in the identification experiment, but AgarAgar (40 µg/g diet powder) was added to slow down diet desiccation. The diet was thoroughly mixed with water (4 ml/g diet powder) adjusted to 90 °C using a thermostat and the resulting slurry was divided into 90 mm petri dishes and allowed to cool down before being offered to the caterpillars. In the SAL-, BEN-, CAT- and MIX-treatments the corresponding supplement was added before introducing the water introduced into the thermostat.

Ten replicate petri dishes of each diet were offered to groups of 7 caterpillars per dish. As we occasionally observed cannibalism during the first few days, we altered the experimental setup after three days: we reduced the number of caterpillars per dish to four and inserted home-made plastic dividers to ensure spatial separation. During this process the caterpillars were re-mixed between the petri dishes within treatments. Diets were changed every third day and caterpillar weights were recorded. After 15 days of adaptation on the diet, the caterpillars were starved for 24 h to allow excretion of residual diet, and then immediately introduced in the conversion experiment.

Isolation and identification of putative metabolites

Two hypothesized metabolites, namely salicin phosphate and catechol glycoside phosphate, were not commercially available and had to be isolated from the feces for structure elucidation. Several grams of feces containing these two metabolites were acquired by feeding 4-5th instar caterpillars diet supplemented with salicin (10 % DW) or catechol (1 % DW). In contrast to HCC, its presumed metabolite catechol is commercially available and was fed instead of HCC-containing salicinoids. LC/MS analysis (data not shown) indicated that the caterpillars produced catechol glycoside and catechol glycoside phosphate no matter if catechol was formed out of salicinoids *in situ* in the gut, or ingested directly as diet constituent. Both phosphates were purified using the same protocol but were isolated separately. Approximately 1 g feces were extracted three times with 5 ml 0.1 molar aqueous NH_3. The extract was applied to a 500 mg Chromabond HR-XA SPE column which was subsequently washed with 5ml water and 5 ml MeOH. Residual washing solvent was removed by aspiration and compounds were eluted three times with 1 ml of a 1 molar NH_4/formate solution in MeOH. The eluate was fractionated on a reversed phase column (EC 250/4.6 Nucleodur Sphinx, RP 5 µm, Macherey-Nagel, Düren, Germany) using a gradient of 20 mmolar ammonium acetate and Acetonitrile (ACN) at 1 ml/min. The ACN proportion was increased from 0 % to 10 % within 10 mins, followed by a 3 min wash with 100 % ACN and a 3 min re-equilibration to 0 % ACN. Salicin phosphate and catechol glycoside fractions were collected from 8:48-9:12 and 9:12-9:22 min, respectively. The solvent was removed using a rotary evaporator and the solid residue was dissolved in a small portion of MeOH. This solution was again fractionated on a reversed phased column (Supelcosil LC-18-DB semi-prep, 25 cm x 10 mm x 5 µm) eluted with 10 mmol ammonium acetate (adjusted to pH=4 with acetic acid) and ACN. The initial proportion of 5 % ACN was increased to 17 % within 6 min, followed by a 2 min wash with 100 % ACN and a two min re-equilibration with 5 % ACN. Salicin phosphate and catechol glycoside

phosphate were both collected from 4:06-4:48 min. The solvent was evaporated to yield approximately 0.5 mg of the respective phosphate, which was analyzed by NMR.

LC/MS (ion trap) analysis

Comparative MeOH feces extracts were analyzed on an Agilent 1100 series chromatograph equipped with a reversed phase column (EC 250/4.6 Nucleodur Sphinx, RP 5 µm, Macherey-Nagel, Düren, Germany) at 25 °C and 0.2 % formic acid and acetonitrile as solvent A and B, respectively. Gradient elution with the following parameters was applied: 0-100 % B (0-50 min), 100 % B (50-55 min), 0 % B (55-60 min). After chromatographic separation analytes where detected using an Esquire 6000 ESI-ion trap mass spectrometer (Bruker Daltonik, Bremen, Germany) operated in alternating ionization mode and scanning a mass range from *m/z*=60-1200. Nitrogen served as nebulizer (35 psi) and drying gas (11 l/min, 330 °C). MS-chromatograms of different diets were compared using the MetaboliteDetect 1.1 Software provided by Bruker Daltonik (Bremen, Germany). This software subtracts a reference chromatogram (e.g. control diet) from a given chromatogram (e.g. salicin diet) and was set to calculate a differential chromatogram showing only *m/z* that had a two times higher intensity than in the reference chromatogram. All salicinoids and their metabolites were readily detected in the negative mode which was also applied for MS3 experiments using the Auto-MS function of the Esquire control software.

NMR analysis

Purified samples were measured using capillary tubes (D_2O, 80 µl filling, 2 mm i.d.) on a Bruker Avance AV500 (Bruker Biospin GmbH, Rheinstetten, Germany) spectrometer equipped with a cryogenically cooled, 5 mm triple channel inverse probe (TCI). Structure elucidation was based on ^{1}H-, ^{13}C-, ^{1}H-^{1}H-COSY, ^{1}H-^{13}C-HSQC and ^{1}H-^{13}C-HMBC NMR spectra. Coupling constants were defined to 145 Hz for $^{1}J_{CH}$ and 10 Hz $^{n}J_{CH}$, respectively. Spectrometer frequencies were 500.130 MHz for ^{1}H resonance experiments and 125.758 MHz for ^{13}C experiments, respectively. For ^{31}P- and ^{1}H-^{31}P-HMBC NMR measurements, a Bruker AV400 spectrometer with a 5 mm BBFO probe was used. Coupling constants were defined to 200 Hz for $^{1}J_{PH}$ and 10 Hz $^{n}J_{PH}$, respectively. ^{31}P chemical shifts were referenced to H_3PO_4 (ξ=0 ppm) as external standard. Spectrometer frequencies were 400.130 MHz for ^{1}H resonance experiments and 161.976 MHz for ^{31}P experiments, respectively. Data acquisition and processing was accomplished using Bruker TopSpin v2.1.

Quantitative chemical analysis

A portion (4-6 mg) of leaf powder was extracted twice with 750 µl MeOH containing 200 µg/l arbutin as internal standard. The combined extracts were 1:20 diluted with pure MeOH and analyzed chromatographically as described below for feces extracts. Feces samples consisted of 2-20 mg and were extracted twice with 50 % aqueous MeOH containing 100 µg/ml arbutin as internal standard. An aqueous solvent was necessary to ensure the quantitative extraction of phosphorylated analytes, which were found to be insufficiently extracted with pure MeOH. Each extraction step was carried out with 5 min ultrasonication followed by 4 min of agitation on a paint shaker. The combined feces extract were diluted 1:20 with pure MeOH and separated on Agilent 1200 series chromatographic system equipped with an Agilent XDB-C-18 column (4.6 x 50 mm, 1.8 µm). Gradient elution with 0.05 % aqueous formic acid (A) and acetonitrile (B) was applied using the following parameters: 5% B (0-0.5 min), 5-70 % B (0.5-4.5 min), 70-100 % B (4.5-6 min), 100 % B (6.0-6.5 min), 5 % B (6.5-10 min). Eluted compounds were detected on an API 3200 LC/MS/MS mass spectrometer (Applied Biosystems, Carlsbad, CA, USA) operated in negative ionization mode and using multiple reaction monitoring (MRM). MRM parameters for each analyte were optimized using commercial standards, compounds kindly provided by Dr. Bernd Schneider, or isolated from caterpillar feces (see above). These standards were also used to generate standard curves referenced to arbutin as internal standard. Concentrations were determined using average response factors calculated from data points within the linear range of the standard curves. MRM-parameters: (parent ion *m/z* → product ion *m/z*; declustering potential [V], collision energy [V]): catechol (109→91; -30, -24), catechol glycoside (270→109; -25, -24) catechol glycoside phosphate (351→79; -30, -52), saligenin (123→93; -20, -12), salicin (285→123; -30, -18), salicin phosphate (365→79; -30, -52), hippuric acid (178→134; -20, -13), salicortin (423→123; -55, -30), tremuloidin (389→121; -20, -26), tremulacin (527→123; -55, -34), arbutin (271→161; -20, -16).

Statistical analysis

We used the open access software package R 2.15.0 for all statistical tests. Weight gain during the adaptation phase was compared using linear mixed effects models (lme). Although data were loglog-transformed, the exponential growth curve of the caterpillars did not permit an analysis over the whole period of the experiment due to the inhomogeneity of variances. Therefore the comparison was restricted to data recorded between day 6 and day 15. Transformed caterpillar weights were nested within petri dishes and treatment and recording day (as factor) entered the model successively as fixed effects and were tested against the null

model. Salicinoid levels in reference and experimental leaf discs were compared using a paired t-test. Treatment related differences of the concentrations of salicinoids, their metabolites and excretion rates were tested using ANOVA after the homogeneity of variances was validated. Percent data (excretion rates) were arcsine transformed. Post-hoc tests were carried out using the Tukey-HSD function.

Results

Identification experiment

The LC/MS chromatograms of caterpillar feces showed that specific *m/z* peaks only occurred when a certain salicinoid was ingested (**Fig. 1**). Salicin was generally found in the feces extract after ingestion of any of the three salicinoids, along with other unknown peaks. Due to the modular composition of salicin, salicortin and tremulacin, an assignment to a specific moiety was easily possible. For example tremulacin is a benzoyl ester of salicortin and any difference between the feces extracts was likely to be a metabolite of the benzoyl moiety. Using this approach a peak with the $[M-H]^-$=365 present in all salicinoid treatments was assigned to the salicin or salicyl alcohol core structure (**Fig. 2**). Two peaks with $[M-H]^-$=271 and 351 were only found in the feces when salicortin or tremulacin were added to the diet, which made them candidates for HCC break down products. Only caterpillars fed with tremulacin excreted a compound with the $[M-H]^-$=178 and this was presumed to be a metabolite of the benzoyl moiety.

In an attempt to gain more structural information on all metabolites, MS3-experiments were carried out. When $[M-H]^-$=365 was fragmented, a neutral loss of 124 mass units ($[M-H]^-$=365 → *m/z*=241) indicated the presence of salicyl alcohol (**Fig. 2**). Further fragmentation (MS3) caused a neutral loss of 162 mass units (*m/z*=241 → *m/z*=79) indicative for glycosidic compounds. This led us to the conclusion that the salicin core structure was still intact and an additional moiety with 80 mass units is attached to the glycone. A prominent *m/z*=97 peak (diagnostic for phosphoric acid) in the MS3 allowed the hypothesis that conjugate 366 is phosphorylated salicin. The elemental composition was confirmed by high resolution mass spectroscopy and NMR-analysis of the purified compound revealed a phosphorylation at the C-3 OH group of the glucose.

Fragmentation of $[M-H]^-$=271 formed a daughter anion with *m/z*=109. We suspected this fragment to be a catechol-like anion, since HCC, the presumed metabolic precursor, has been

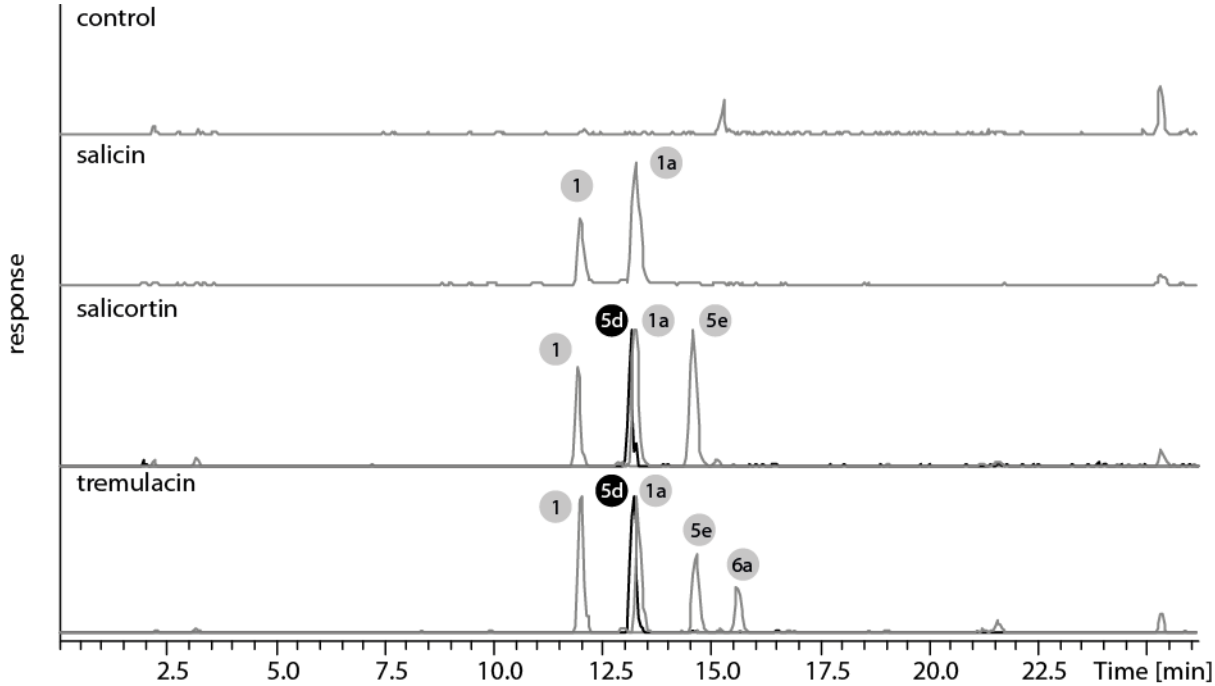

Figure 1 Chromatograms of MeOH feces extracts of *Lymantria dispar* fed with different artificial diets. Extracted ion chromatograms on the *m/z*=285, 365, 271, 351 and 178, which correspond to the $[M-H]^-$ of salicin (1), salicin phosphate (1a), catechol glycoside (5d), catechol glycoside phosphate (5e) and hippuric acid (6a). Note that salicin phosphate (1a) and catechol glycoside (5d) are co-eluting. Structures with identical numbering are shown in Figure 5.

repeatedly suggested to form catechol (Ruuhola, Tikkanen & Tahvanainen 2001). The neutral loss of 162 mass units (*m/z*=271 → *m/z*=109) again suggested a glycosidic nature and therefore catechol glycoside was hypothesized as candidate structure. In analogy to the previously found salicin phosphate, catechol glycoside was proposed to form catechol glycoside phosphate. The hypothetical mass of this phosphate matched the second new peak ($[M-H]^-$=351) and the elemental composition was confirmed by high resolution mass spectrometry. Catechol glycoside phosphate was isolated from feces of *L. dispar* fed with catechol supplemented diet and NMR analysis revealed again a phosphorylation of the C-3 atom OH group.

The odd molar weight of $[M-H]^-$=178 and a good positive mode ionization made the presence of nitrogen obvious. Therefore we suspected a glycine conjugate of benzoic acid (i.e. hippuric acid), which was proven by comparison with a commercial standard.

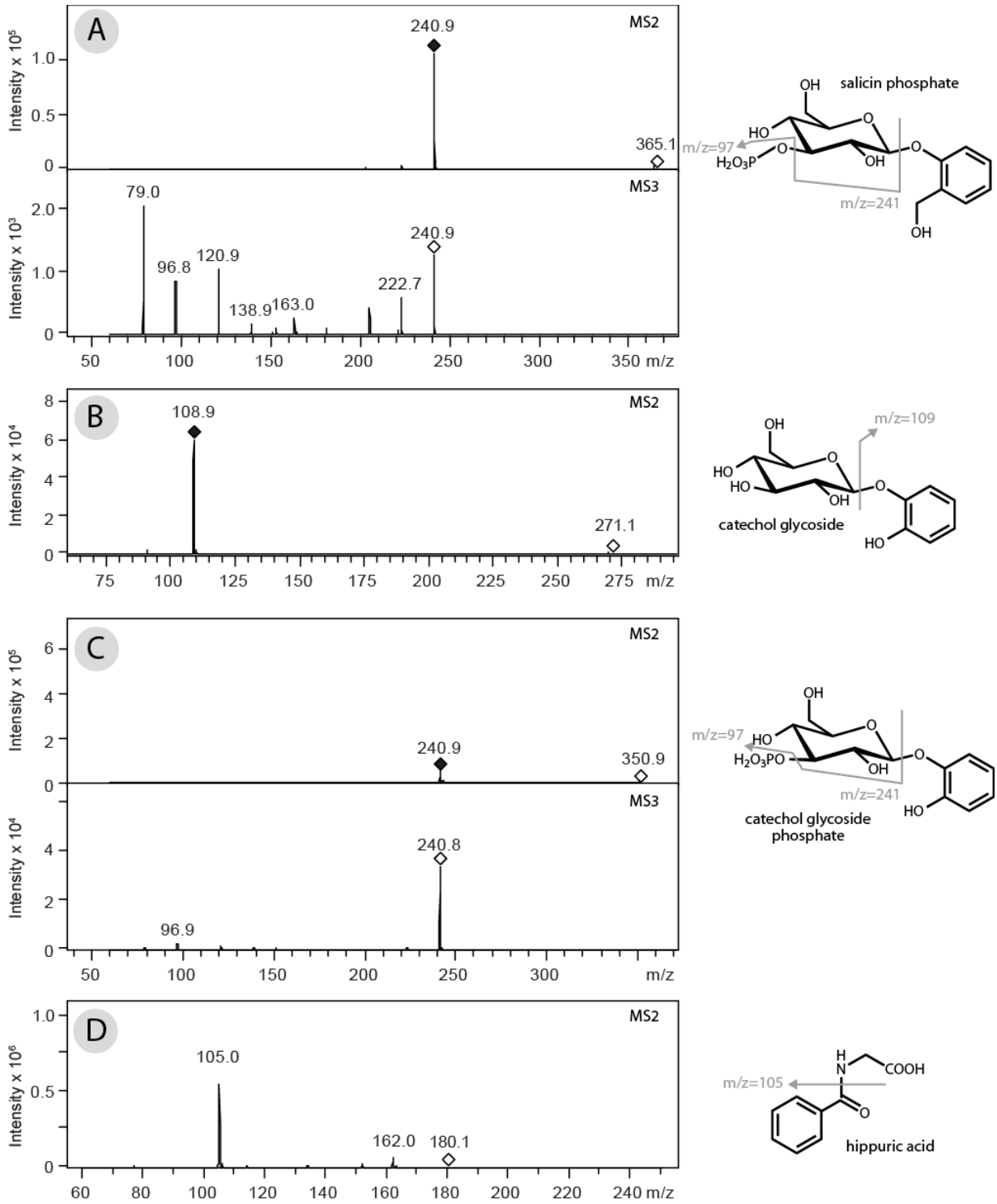

Figure 2 MSn spectra of four new salicinoid metabolites found in *Lymantria dispar* feces extracts (A salicin phosphate, B catechol glycoside, C catechol glycoside phosphate and D hippuric acid). Panels on the right show proposed fragmentation and the corresponding peaks in mass spectra. All compounds except hippuric acid were measured in negative mode.

Conversion experiment

In this experiment we determined the excretion rates of salicinoids contained in *P. tremula x tremuloides* leaf discs into the metabolites described above. Analysis of the reference leaf discs revealed that salicortin (1.9 % DW) and tremulacin (1 % DW) were the major salicinoid constituents in addition to minor amounts of salicin (0.1 % DW) and tremuloidin (0.02 % DW, data not shown). These foliar concentrations were much lower than the levels in the salicin-, catechol-, benzoic acid- and mixed-diets that were used during the preconditioning. Interestingly we also found a small amount of foliar catechol glycoside (0.01 % DW), which may originate from the tissue damaged at the edge of the leaf discs. When the salicinoid composition of reference leaf discs (sampled at the beginning of the experiment) and experimental leaf discs (sampled after 18 h of caterpillar feeding) were compared, no significant differences in concentrations of salicinoid components (i.e. salicin, HCC and benzoyl moieties) were found (**Supplemental 1**), and therefore the concentrations of experimental leaf discs were used for the calculation of excretion rate calculation.

At the beginning of the conversion experiment, the caterpillar weights differed vastly between treatments as a result of the preconditioning (see performance data below), but this had no significant effect on the amount of ingested leaf material (ANOVA $p=0.268$, data not shown). In contrast, the diet pre-treatment during the adaptation phase had some impact on the metabolite concentrations in the feces (**Supplemental 2**). Fecal metabolite concentrations, however, are not ideal measures for the detoxification activity, since a given amount of a metabolite is diluted in a variable amount of feces dependent on food conversion. Therefore excretion rates (i.e. percentage of ingested material recovered in the feces) were calculated for ingested dry matter (in mg) and the three salicinoid aglycones (in mol). Overall, the dry matter excretion rate was not significantly affected by the treatment, although the caterpillars reared on the catechol- and mixed-diet appeared to use their food more efficiently (50-55 % excretion rate) than their conspecifics reared on the other diets (excretion rate 70-80 %, **Fig. 3**). In contrast, the treatment significantly influenced the excretion rates of the two phosphorylated compounds (both $p<0.001$) and hippuric acid ($p=0.014$), but not of catechol glycoside and salicin. Catechol glycoside phosphate and salicin phosphate excretion was much lower when the caterpillars were previously fed on catechol-, salicin- and mixed-diets, while the two metabolites were virtually absent in the poplar-treatment. Small amounts were detected when the feces extracts were injected undiluted, but quantification was not possible. Fecal hippuric acid excretion did not show a clear cut pattern but was high for the control and benzoic acid treatment and particularly low for the catechol treatment. In summary, only 10 % of the

ingested HCC were recovered in the form of catechol glycoside or catechol glycoside phosphate, while more than 50 % of the ingested salicyl alcohol moieties were found in the feces as salicin or salicin phosphate. Up to 75 % of the ingested benzoic acid was eliminated through the feces as hippuric acid.

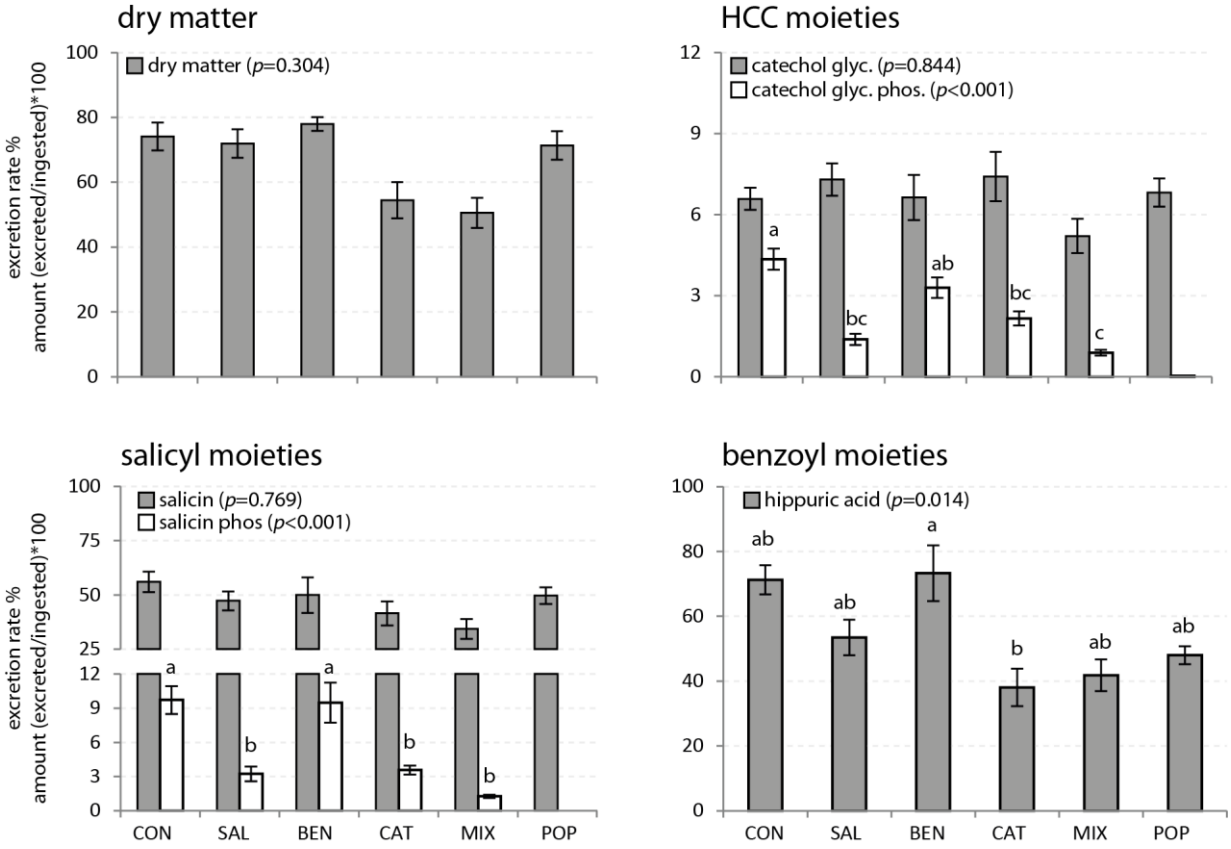

Figure 3 Excretion rates of salicinoids in *Lymantria dispar* caterpillars after different diet pre-treatments. X-axis indicates the diet supplements used in the pre-treatment: CON=control, SAL=salicin, BEN=benzoic acid, CAT=catechol, MIX=mixture of salicin, catechol and benzoic acid, POP=poplar leaves. Excretion rates were calculated as the amount (in gram or mol) 100 x excreted/ingested. P-values indicate comparison of treatments based on one-way ANOVA. Small letters indicate the result of Tukey-HSD post-hoc tests.

Performance experiment

This experiment aimed to evaluate the physiological impact of salicinoid components in a 15 day feeding trial on different diets. During the first 3 days several incidences of cannibalism occurred (especially on the salicin-, catechol and mixed-diets) until the caterpillars were spatially divided. In the course of the experiment, the gypsy moth weights (**Fig. 4**) were significantly influenced by time and the diet the caterpillar were fed (both $p<0.001$). Generally, the weight gain was highest on benzoic acid- and control-diets, intermediate on salicin- and catechol-diets and lowest on mixed- and poplar-diets. The comparison of diets only within these three blocks showed that the weight gain on the benzoic acid-diet was higher compared

to the control-diet (p=0.0294 BEN vs CON), while salicin- and catechol-diets were not different from each other (p=0.642 SAL vs CAT). Growth rates on poplar leaves were even lower than on the mixed-diet (p=0.0025). Survival (excluding cannibalism) was similarly high on the catechol-, salicin-, benzoic acid, and control-diets, where only very few caterpillars died. *L. dispar* on the mixed-diet had a significantly lower but still intermediate survival compared to individuals reared on poplar leaves. However, fatalities on the poplar-diet were occasionally due to infestation with a polyhedrosis virus and are therefore not comparable to the other treatments.

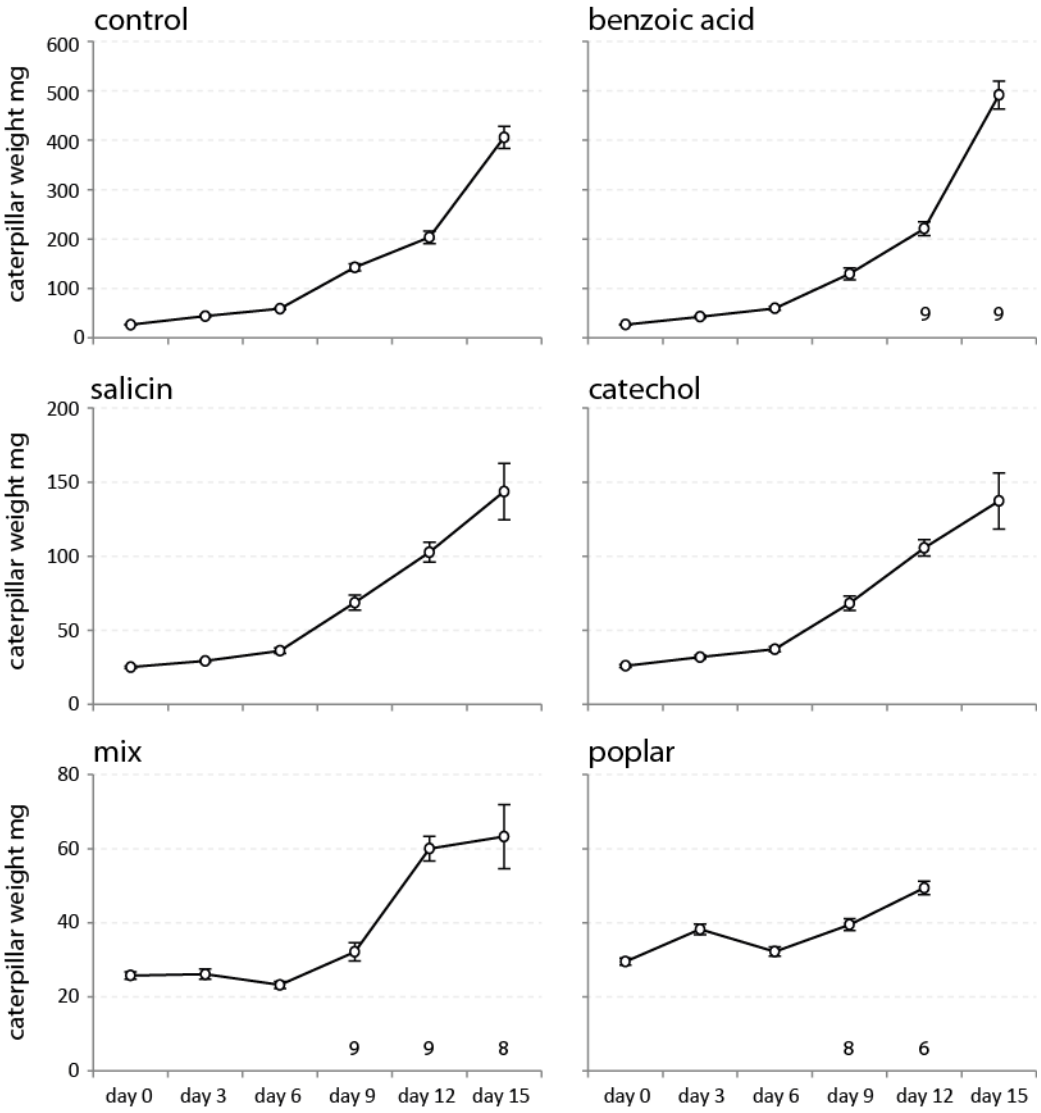

Figure 4 Growth curves of *Lymantria dispar* caterpillars on different diets during the adaptation experiment. Data points indicates the average ± SE of ten petri dishes with four individual caterpillars each. When the number of replicates was lower than 10, the actual value is displayed above the x-axis. Note different y-scaling between treatments.

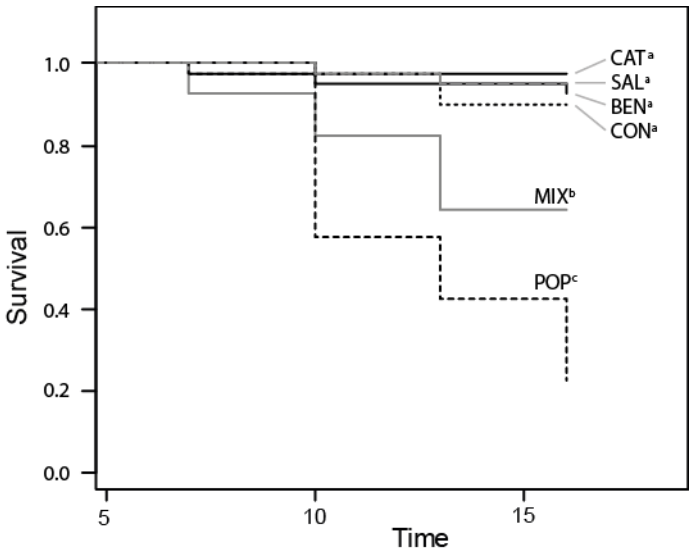

Figure 5 Kaplan-Meyer survival curves of *Lymantria dispar* caterpillars reared on diets containing different salicinoid components (CAT=catechol, SAL=salicin, BEN=benzoic acid, CON=control, MIX=catechol+salicin+benzoic acid, POP=poplar leaves). Superscript letters indicate groups of similar survival as found by model simplification.

Discussion

Numerous bioassays have shown the impact of salicinoids on fitness parameters of generalist herbivores, such as mortality, growth, pupal weight and time to pupation (Lindroth, Scriber & Hsia 1988; Lindroth & Bloomer 1991; Hemming & Lindroth 1995; Ruuhola, Tikkanen & Tahvanainen 2001; Boeckler *et al.* 2014). However, the metabolic processes in the insect gut and the fate of individual plant metabolites are still a matter of speculation and various theories need validation (Lindroth, Scriber & Hsia 1988; Clausen, Keller & Reichardt 1990; Ruuhola, Julkunen-Tiitto & Vainiotalo 2003). Our investigations of salicinoid metabolism in *L. dispar* showed that the salicin core structure of salicin (**1**), salicortin (**2**) tremuloidin (**4**) and tremulacin (**3**) stays intact while the ester bonds in salicortin and tremulacin are quantitatively degraded during passage through the digestive tract. The released salicinoid components were partly recovered as conjugates. Two of these conjugates contained phosphorylated sugars, and their formation was dependent on preliminary exposure to phenolics. Our caterpillar performance experiment demonstrated that some salicinoid degradation products have a negative effect on caterpillar growth and survival when supplemented to artificial diet in genuine form.

Salicinoid degradation in the gypsy moth

Similar to a study with *Operophtera brumata* (Ruuhola, Tikkanen & Tahvanainen 2001) we observed that the salicin core structure present in all salicinoids largely stays intact during

digestive transit of gypsy moth caterpillars, while the ester bonds in higher order salicinoids are quantitatively degraded (**Fig. 5**). The basic conditions in the gut of gypsy moth and many lepidopteran caterpillars (Johnson & Felton 1996) certainly favor a spontaneous, non-enzymatic ester cleavage and *in vitro* studies have shown that complex salicinoids are labile at alkaline pH (Ruuhola, Julkunen-Tiitto & Vainiotalo 2003). We thus conclude that the first steps of salicinoid degradation do not necessarily require plant indigenous enzymes, as is the case for other glycosidic defense compounds (Pentzold *et al.* 2013). The structures of the conjugates observed in gypsy moth feces provide evidence of the further metabolic fate of the HCC and benzoic acid released from salicortin and tremulacin, respectively. The metabolism of the liberated HCC (**5**) has been predicted earlier to lead to the formation of catechol (**5d**) after decarboxylation (Clausen *et al.* 1989; Julkunentiitto & Meier 1992) and our finding of catechol glycosides (**5e**) corroborates this hypotheses. Unlike Ruuhola, Tikkanen and Tahvanainen (2001), we were not able to detect catechol in the feces directly, but the glycoside is likely to be a detoxification product of the transiently formed free di-phenol. In contrast to HCC, benzoic acid is apparently not subjected to further transformations and directly conjugated. Based on these findings and complimentary to the previous literature we suggest novel metabolic routes of salicinoids in generalist lepidopteran herbivores (**Fig. 5**).

Detoxification activities in the gypsy moth

Our feces screening showed that salicinoid metabolites are excreted as glycine conjugates (benzoic acid), glycosides or phosphorylated glycosides (salicin and catechol). Glycosylation of xenobiotics have been observed in *Bombyx mori* (Luque, Okano & O'Reilly 2002), *Mythimna separata* (Sasai *et al.* 2009) and *Manduca sexta* (Ahmad & Hopkins 1993) and are typically catalyzed by UDP-glycosyltransferases, a very diverse multigene family in insects (Ahn, Vogel & Heckel 2012) and other organisms. The closely related glucuronosyltransferases are often used by vertebrates to catalyze the corresponding conjugation with glucuronic acid and such a reaction has been observed in possum for salicin and salicyl alcohol (McLean *et al.* 2001). The deployment of glucosidases with broad substrate specificity may enable *L. dispar* and other generalists to cope with plant toxins from a broad range of host plants. Surprisingly, small proportions of both salicin and catechol glycoside are also phosphorylated before excretion to form a so far unknown form of glucose phosphate conjugates (**1a** and **5e**). As the glycosides appear to be relatively stable species that can be eliminated through the feces, a further metabolic conversion at the cost of phosphorus is unexpected.

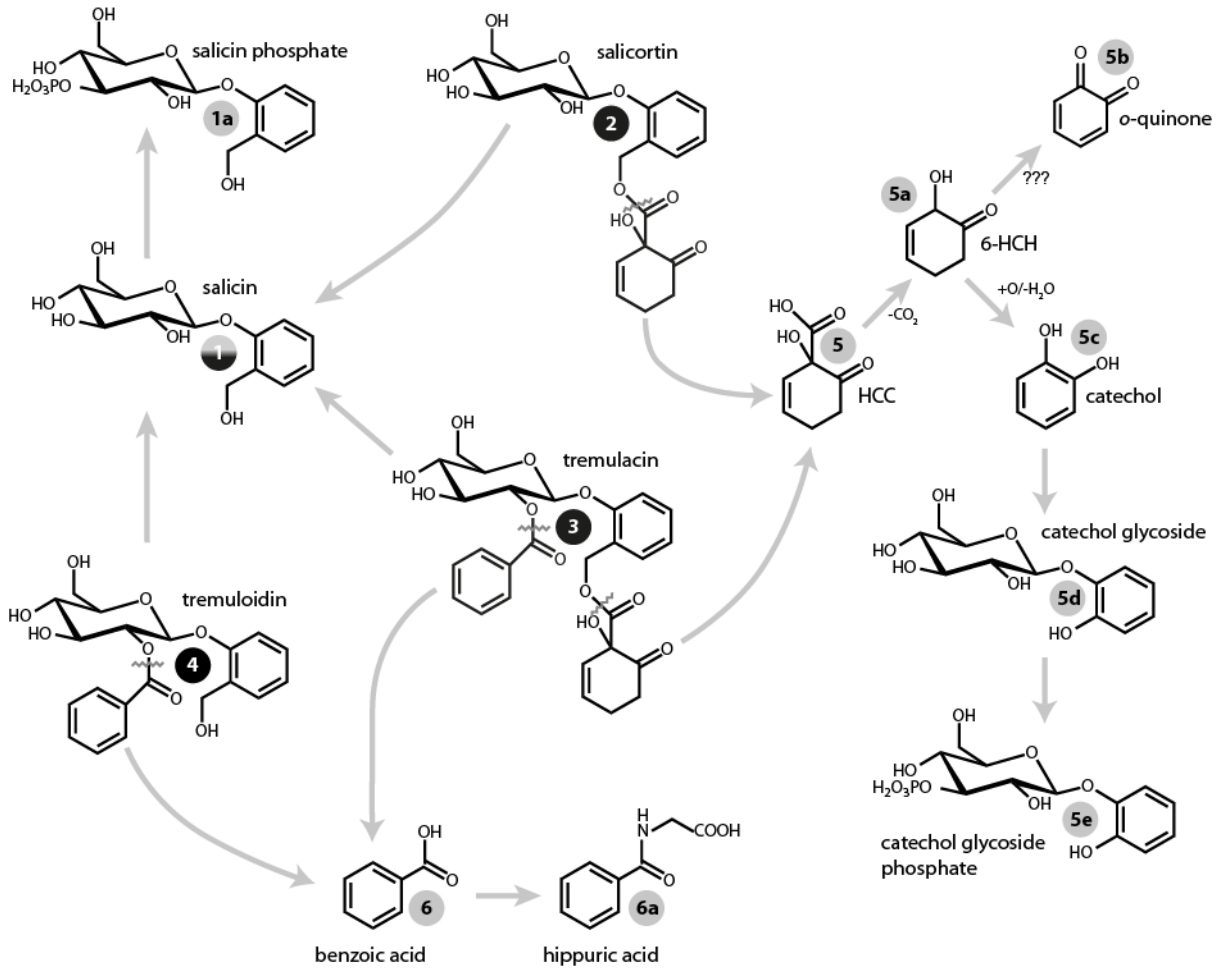

Figure 6 Suggested metabolic routes for the salicinoid breakdown products observed in *Lymantria dispar* feces. Colored numbering indicates if compounds are produced by the plant (black) or in the caterpillar (grey). Salicin (1) can be plant derived or a metabolite of higher order salicinoids.

We speculate that the phosphorylation serves as metabolic tag that marks certain glycosides for transport processes or makes them unsuitable substrates for glucosidases. In contrast to the phosphorus-containing metabolites, the glycine conjugate hippuric acid (**6a**) is a well-known metabolite of benzoic acid (**6**) in vertebrates (Marsh, Wallis & Foley 2005; Beyoglu & Idle 2012) and insects (Shyamala 1964; Nijhout 1975). Konno, Hirayama and Shinbo (1996) observed that some Lepidoptera contain unusually high levels of free glycine and suggested that this may be due to its role in the conjugation of dietary aromatic acids. Besides the metabolic cost of glycine, the formation of hippuric acid from tremulacin gives no explanation why this salicinoid is more toxic than salicortin (which contains no benzoic acid), as has been observed earlier (Lindroth, Scriber & Hsia 1988).

Further investigations are required to re-evaluate this phenomenon and to unravel the possible synergistic toxicity when different conjugation pathways are challenged simultaneously. Moreover, it has to be investigated if the current models of salicinoid breakdown apply to other herbivores. As we suggest that the ester bonds are mainly degraded non-enzymatically due to

the basic gut pH in *L. dispar*, it is important to elucidate the processes in other generalists and specialists with neutral gut conditions.

Conversion efficiency of salicinoid components

The recovery of salicinoid moieties in the form of fecal metabolites increased considerably in the order HCC<salicin<benzoic acid when *L. dispar* was fed *P. tremula x tremuloides* leaves. Only 10 % of the ingested HCC were found as conjugates in the feces, while salicin (approx. 60 %) and benzoic acid (approx. 75 %) were excreted more efficiently. The residual, non-recovered fractions of each moiety must have been transformed in different ways. One possible sink is the reaction with biological targets, such as the hypothesized addition of catechol-derived *o*-quinones to proteins (Appel 1993). Alternatively, the metabolites may have been detoxified in other ways or were resorbed and transported to other organs. Such metabolic processes have rarely been elucidated for secondary metabolites in insect herbivores. One exception are certain poplar and willow feeding leaf beetles, which sequester host derived defense compounds employing highly specialized metabolic conversion, transport and storage capabilities (Strauss *et al.* 2013). If the phosphorylated glucose conjugates in *L. dispar* are destined for transport or export, the significant reduction of these compounds in the feces of *L. dispar* larvae pre-conditioned on phenolic-containing diets may indicate a metabolic adaptation leading to a lower uptake of toxins. More research is needed, but the *in vivo* tracking of salicinoid products and other harmful metabolites is very challenging due to the multiplicity of possible physiological targets. While radio-isotope labeled compounds allow the observation of the metabolic fate of compounds in organisms, this method provides no information about the chemical species detected. So far, only Schramm *et al.* (2012) could show in a combined approach with radio-labeled and stable isotope-labeled precursors that generalist *Spodoptera littoralis* caterpillars almost quantitatively excretes glucosinolates as isothiocyanates or their amino acid conjugates. However, it appears unlikely that the toxicity residing in the glucosinolates merely originates from the metabolic cost of conjugation, so even for this well-studied group of plant compounds, no definite *in vivo* targets were identified so far.

Bioactivity of salicinoid components

The adverse effects of salicinoid breakdown products are supported by our performance study, in which we demonstrated that growth inhibition was high for catechol and this molecule is only conjugated to a small extent. Although it is currently unclear if the breakdown of HCC to catechol is quantitative, pure catechol had the strongest growth inhibitory effect of the three

salicinoid moieties considering it was administered at lower amounts (150 µmol/g DW) than salicin (200 µmol/g DW). It is well known that salicortin and tremulacin, two HCC containing salicinoids, are especially effective in reducing larval and pupal weights and increasing mortality and instar duration (manuscript I). Such physiological effects are likely the consequence of insufficient detoxification as found in our study. Surprisingly, salicin had the same effect on caterpillar weight gain at the dose administered in our experiment, although this compound was reported to have little effect in other studies (Lindroth 1988a; Lindroth & Peterson 1988). In previous studies, salicin was typically offered in doses matching the low foliar concentration of many Salicaceae. We fed salicin at a comparatively high dose (200 µmol/g=5.7 % DW) to simulate the amount liberated from all dietary salicinoids in the caterpillar gut (*vide supra*) and this possibly led to a stronger effect. It is currently unclear how salicin could harm *L. dispar*, but it is conceivable that herbivores reduce their glucosidase activity to avoid salicin cleavage, as observed by Lindroth (1988b), and therefore suffer from a lower absorption of dietary sugars. In this respect it is noteworthy that only the mixed diet containing salicin, catechol and benzoic acid led to an increase in mortality of *L. dispar* (**Fig. 5**). While benzoic acid was certainly easily conjugated, the necessity to glucosylate catechol, avoiding enzymatic salicin cleavage, and digesting dietary sugars may have led to an unresolvable dilemma for the glucosylation/deglucosylation system. In any case, our growth curves suggest that *L. dispar* tolerated high amounts of dietary phenolics in the artificial diet, which is supposedly rich in nutrients. Growth was significantly lower on poplar leaves with lower salicinoid concentrations but also likely lower levels of nutrients. This and the infestation with a polyhedrosis virus render the caterpillars fed with foliage not directly comparable with the treatments on artificial diet. Future experiments should investigate how the availability of nutrients interacts with the adverse effect of salicinoids in insect herbivores. Such studies should especially consider elements or molecules that are required for the detoxification, such as glucose, glycine and phosphorus.

In summary, we identified five conjugates of salicinoid breakdown products in generalist *L. dispar* caterpillars. Two of the compounds observed were so far unrecognized phosphorylated sugar derivatives of unknown function. Excretion rates of the five metabolites varied and were influenced by previous nutrition. Conversion rates appeared to be negatively correlated with performance, indicating that unconverted molecules interfered with the caterpillar's metabolism. Much additional research is still needed to determine how secondary metabolites are processed in herbivores, and the basis of their toxicity. Our preliminary findings on the activation, conjugation and excretion of salicinoids are important milestones to better

understand the first steps in this frontline of plant-insect interactions: the molecular interface between plant material and insect tissue in the gut.

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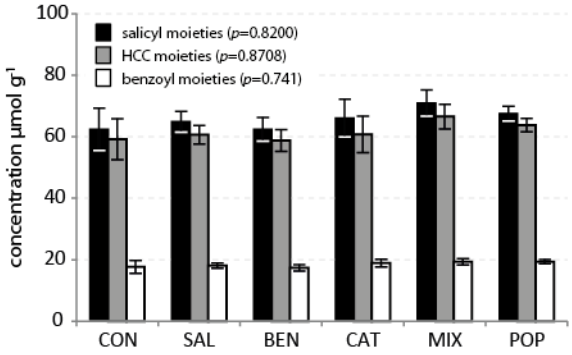
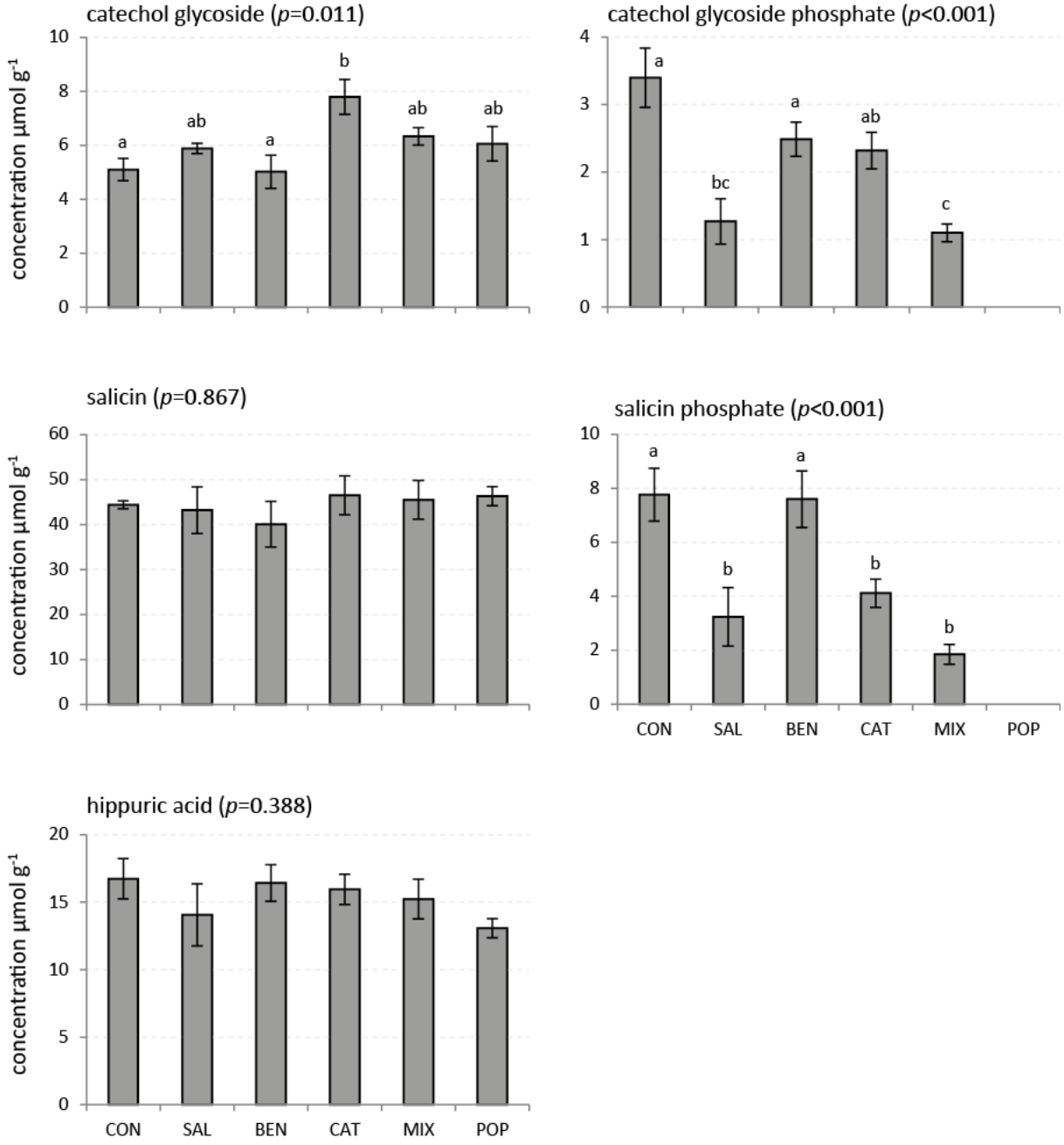
Appendix

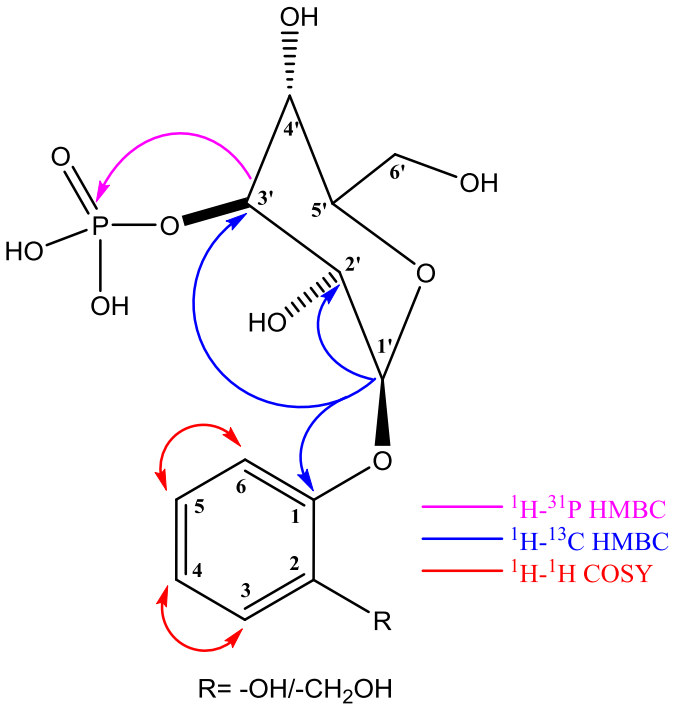
Supplemental 1: Concentrations of salicinoid building blocks in *Populus tremula x tremuloides* leaf discs. Bars show Mean ± SE.



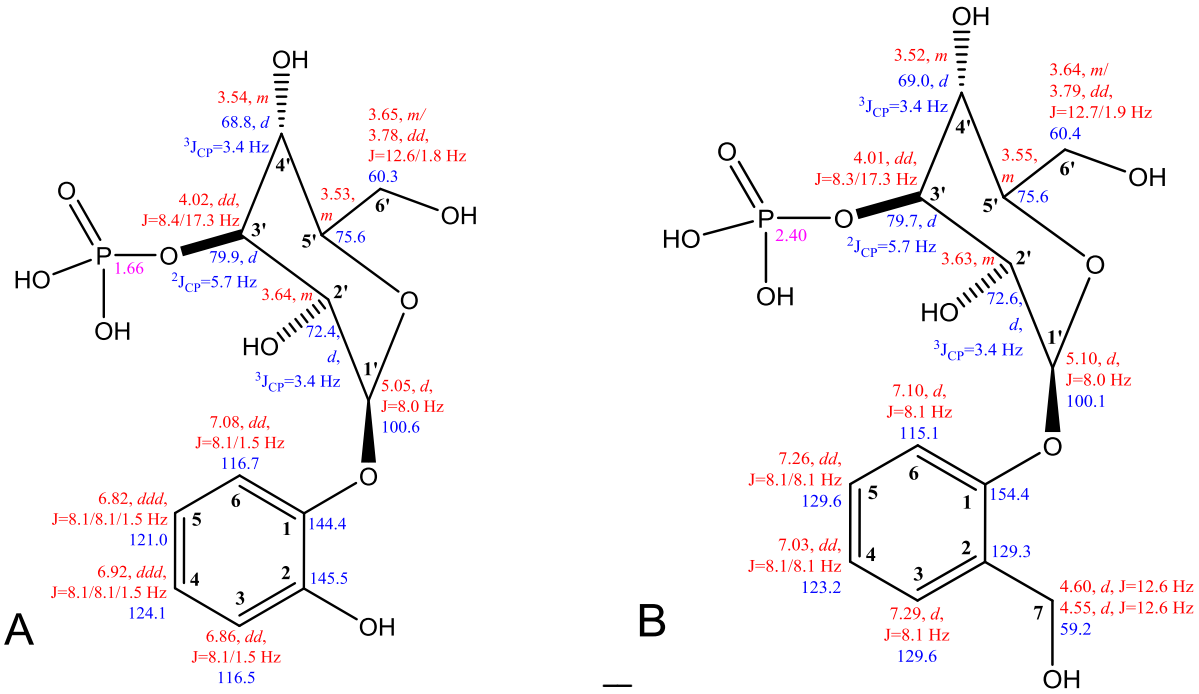
Supplemental 2: Concentrations of salicinoid metabolites in the feces of *Lymantria dispar* caterpillars reared on different diets. Bars show Mean ± SE. P-values of one-way ANOVA are given above the chart titles. Small letters indicate the result of a Tukey-HSD post-hoc test.



Supplemental 3: Key correlations for elucidation of catechol glycoside phosphate and salicin phosphate.



Supplemental 4: Structures of catechol glycoside phosphate (A) and salicin phosphate (B) including chemical shift data.



Supplemental 5: ^{1}H- and ^{13}C-NMR data of catechol glycoside phosphate and salicin phosphate in D_2O.

		catechol glycoside phosphate					salicin phosphate				
No.		δ_C	δ_H	δ_P	J_{HH} [Hz]	J_{CP} [Hz]	δ_C	δ_H	δ_P	J_{HH} [Hz]	J_{CP} [Hz]
1	**C**	144.4	-	-	-	-	154.4	-	-	-	-
2	**C**	145.5	-	-	-	-	129.3	-	-	-	-
3	**CH**	116.5	6.86	-	*dd*, 8.1/1.5	-	129.6	7.29	-	*d*, 8.1	-
4	**CH**	124.1	6.92	-	*ddd*, 8.1/8.1/1.5	-	123.2	7.03	-	*dd*, 8.1/8.1	-
5	**CH**	121.0	6.82	-	*ddd*, 8.1/8.1/1.5	-	129.6	7.26	-	*dd*, 8.1/8.1	-
6	**CH**	116.7	7.08	-	*dd*, 8.1/1.5	-	115.1	7.10	-	*d*, 8.1	-
7a **7b**	**CH_2**	-	-	-	-	-	59.2	4.60 4.55	-	*d*, 12.6 d, 12.6	-
1'	**CH**	100.6	5.05	-	*d*, 8.0	-	100.1	5.10	-	*d*, 8.0	-
2'	**CH**	72.4	3.64	-	*m*	*d,* 3.4	72.6	3.63	-	*m*	*d,* 3.4
3'	**CH**	79.9	4.02	-	*dd*, 8.4/17.3	*d,* 5.7	79.7	4.01	-	*dd*, 8.3/17.3	*d,* 5.7
4'	**CH**	68.8	3.54	-	*m*	*d,* 3.4	69.0	3.52	-	*m*	*d,* 3.4
5'	**CH**	75.6	3.53	-	*m*	-	75.6	3.55	-	*m*	-
6'a **6'b**	**CH_2**	60.3	3.65 3.78	-	*m* *dd*, 12.6/1.8	-	60.4	3.64 3.79	-	*m* *dd*, 12.7/1.9	-
	PO_4	-	-	1.66	-	-	-	-	2.40	-	-

3.4. Manuscript IV: Gypsy moth feeding has only marginal impact on phenolic compounds in old-growth black poplar

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Gypsy Moth Caterpillar Feeding has Only a Marginal Impact on Phenolic Compounds in Old-Growth Black Poplar

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Abstract Species of the Salicaceae produce phenolic compounds that may function as anti-herbivore defenses. Levels of these compounds have been reported to increase upon herbivory, but only rarely have these changes in phenolics been studied under natural conditions. We profiled the phenolics of old-growth black poplar (*Populus nigra* L.) and studied the response to gypsy moth (*Lymantria dispar* L.) herbivory in two separate field experiments. In a first experiment, foliar phenolics of 20 trees were monitored over 4 weeks after caterpillar infestation, and in a second experiment the bark and foliar phenolics of a single tree were measured over a week. Of the major groups of phenolics, salicinoids (phenolic glycosides) showed no short term response to caterpillar feeding, but after 4 weeks they declined up to 40 % in herbivore damaged and adjacent undamaged leaves on the same branch when compared to leaves of control branches. Flavonol glycosides, low molecular weight flavan-3-ols, and condensed tannins were not affected by herbivory in the first experiment. However, in the single-tree experiment, foliar condensed tannins increased by 10–20 % after herbivory, and low molecular weight flavan-3-ols decreased by 10 % in the leaves but increased by 10 % in the bark. Despite 15 % experimental leaf area loss followed by a 5-fold increase in foliar jasmonate defense hormones, we found no evidence for substantial induction of phenolic defense compounds in old growth black poplar trees growing in a native stand. Thus, if phenolics in these trees function as defenses against herbivory, our results suggest that they act mainly as constitutive defenses.

Keywords Field experiment · Foliar chemistry · Induction · Plant insect interaction · Salicinoids (phenolic glycosides) · Secondary metabolites

Introduction

Phenolic secondary metabolites are produced by many plant species and appear to be important in defense against herbivores and pathogens (Appel 1993; Barbehenn and Constabel 2011; Boeckler *et al.* 2011). Much of the evidence for their defensive role comes from correlative studies of plant phenolic content and the ability to resist herbivores and pathogens (Bryant and Kuropat 1980; Hwang and Lindroth 1997; Mutikainen *et al.* 2000), but more recent mechanistic studies have increased our understanding of their mode of action against plant enemies (Barbehenn *et al.* 2010).

Among the most prolific producers of phenolics are trees of the family Salicaceae. Species of poplar and willow are especially rich in two groups of phenolics: salicinoids, commonly called phenolic glycosides, and flavan-3-ol oligomers, also called condensed tannins or proanthocyanidins. We prefer the term salicinoids instead of phenolic glycosides to avoid confusion with the flavonol glycosides, which in chemical terms are also phenolic glycosides. Salicinoids are structural derivatives of salicin, the 1-O-β-glycoside of salicyl alcohol, and they are typical of the Salicaceae. Complex salicinoids are esters of salicin and organic acids (*e.g.* benzoic acid). These compounds often are species-specific (Julkunen-Tiitto 1985) and occur in concentrations of at least 1 % leaf dry weight (Thieme and Benecke 1971). Salicinoids negatively impact the performance of generalist herbivores like *Lymantria dispar* and *Malacosoma disstria* (Lindroth 1991).

Condensed tannins (CT) are oligomers of C-C linked flavan-3-ol subunits with a variable chain length (Ayres *et al.* 1997; Scioneaux *et al.* 2011). They usually are the most

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abundant phenolic compounds in Salicaceae species and can comprise up to 30 % of the leaf dry weight (Donaldson *et al.* 2006). Despite their abundance, the impact of CT on herbivores still is unclear. Many insect herbivores are not affected by CT (Ayres *et al.* 1997; Hemming and Lindroth 1995), but these compounds may play an important role in deterring mammalian herbivores (Heiska *et al.* 2007; Spalinger *et al.* 2010) and in pathogen defense (Holeski *et al.* 2009; Miranda *et al.* 2007). Salicaceae also produce minor amounts of monomeric and dimeric flavan-3-ols (low molecular weight flavan-3-ols), but these compounds also lack a detrimental effect on leaf feeding herbivores (Barbehenn *et al.* 2008). Besides salicinoids and CT, small amounts of phenolics such as flavonol glycosides (*e.g.*, quercetin glycosides) and phenolic acids (*e.g.*, caffeic acid derviatives) have been reported in the Salicaceae (Palo 1984), and some of these also may function in herbivore defense (Lindroth and Peterson 1988).

Phenolic compounds affect the herbivores that naturally occur on Salicaceae and *vice versa*. Climate chamber, green house, and common garden experiments have shown that levels of salicinoids and CT in Salicaceae species may increase when foliage is damaged mechanically or by herbivores (Fields and Orians 2006; Peters and Constabel 2002; Ruuhola *et al.* 2001; Stevens and Lindroth 2005). The "induction" of secondary metabolites by herbivory is considered a cost-saving strategy since allocation of resources to defenses only happens when plants are challenged by herbivores (Zangerl 2003). The process is initiated by a complex signal cascade that increases signaling molecules, such as jasmonic acid and other hormones, leading to the production of defense compounds. The induction of CT in Salicaceae has been observed regularly within 3–7 days following mechanical or herbivore damage, and has been demonstrated at the phenotypic level (Osier and Lindroth 2001; Stevens and Lindroth 2005) and by gene expression analysis of key enzymes in CT biosynthesis (Mellway *et al.* 2009; Peters and Constabel 2002). In contrast, the numerous studies on salicinoid induction (*e.g.*, Clausen *et al.* 1989; Fields and Orians 2006; Lindroth and Kinney 1998; Ruuhola *et al.* 2001; Stevens and Lindroth 2005; Young *et al.* 2010) have not revealed any consistent time course, and induction has been observed at intervals ranging from 24 h (Clausen *et al.* 1989) to 8 weeks after the stimulus (Stevens and Lindroth 2005). In addition, some researchers have reported induction only in leaves distal to the actual site of damage (Fields and Orians 2006; Ruuhola *et al.* 2001; Stevens and Lindroth 2005), whereas others have observed induction in the damaged tissue itself (Clausen *et al.* 1989; Lindroth and Kinney 1998; Young *et al.* 2010). Still other studies have found no clear evidence for salicinoid induction at all (Julkunen-Tiitto *et al.* 1995; Osier and Lindroth 2001). More work on the response of salicinoids to herbivores is necessary to resolve these issues.

The majority of studies on salicinoid induction have been conducted with *Populus tremuloides* although it is well known that Salicaceae differ widely with respect to their salicinoid profile (Boeckler *et al.* 2011). In addition, less attention has been paid to phenolic compounds other than salicinoids and CT. Almost all of the experiments on phenolic induction have been carried out under controlled laboratory and greenhouse conditions and have utilized young trees. It is important to know if the trends reported are valid under natural conditions, especially in older trees.

Here, we investigated the effect of herbivory on the major phenolics of *Populus nigra* (black poplar) under field conditions. Black poplar is native to Europe, Asia, and northern Africa, and rarely has been the subject of plant-herbivore or pharmacological studies. Knowledge on the phenolic compounds present in this species is scant, and thus, a thorough characterization of the phenolic chemistry was necessary. The relationship between experimental herbivory mediated by gypsy moth (*Lymantria dispar*) caterpillars, a polyphagous herbivore of poplars in Europe and northern Asia, was examined in two separate field experiments, one with 20 *P. nigra* trees of separate genotypes (2009) and another with a single tree (2010). In the 2010 experiment, we also quantified defense related hormones in order to evaluate responses in the context of a natural background of pre-existing herbivory.

Methods and Materials

Study Site The study was conducted on representatives of a natural population of *Populus nigra* located in a floodplain forest on an island in the Oder river near Küstrin-Kietz, Germany (52°34'1"N, 14°38'3"E, elevation: approx. 20 m above sea level). Periodic flooding gives the site the typical landscape of a river floodplain with a scattered woody vegetation consisting mainly of *P. nigra*, *Salix* spp., and *Quercus* spp. trees. The *P. nigra* population consists of 3~50 individuals with equal sex distribution. In 2009–2010, the mean annual air temperature was 11.5 °C and the annual precipitation was 402.3 mm.

Insects Lymantria dispar eggs were provided by the United States Department of Agriculture, Animal and Plant Health Inspection Service (Buzzards Bay, MA, USA). Larvae were reared in a climate chamber (25 °C, 60 % humidity, 14:10 L:D period) on artificial gypsy moth diet (MP Biomedical, Eschwege, Germany). After reaching the 4th instar, individual caterpillars were separated in plastic cups (Solo Cup Co., Highland Park, IL, USA) and starved for 24 h before use in an experiment.

2009 Experiment – Effect of Herbivory on Phenolics in 20 Trees The study was conducted between the 11th of August

2009 and the 9th of September 2009 in 20 old-growth black poplar trees (10 female, 10 male) with an approximate average height of 20–30 m and an age of 30–60 years. Given the timing of gypsy moth abundance reported in Germany (Ebert 1994), older larvae (≥4th instar) can be expected to occur in the field from April until August. Six branches per tree at a height of 1–2 m were selected as experimental branches. Mesh cylinders (thread thickness 0.15 mm, mesh size 1 mm) were installed on experimental branches to enclose all leaves located within 20–60 cm of the branch apex. Depending on the internode length and shoot growth of the specific tree, approximately 15–25 leaves were enclosed by the mesh bag, while 8–14 unenclosed leaves remained between the mesh bag and the branch apex. In three of the six mesh bags per tree, five gypsy moth caterpillars of the 4th instar were released, and these branches were designated as herbivory treatments. Branches with empty mesh bags served as control treatments. For the remainder of this paper, we refer to the leaves that were covered with mesh bags in the first 48 h of the experiment as "enclosed leaves" and the leaves immediately adjacent to those in the apical direction on the branch the "adjacent leaves" (Supplemental Fig. 1). Caterpillars readily fed on enclosed foliage, and preliminary experiments showed that they developed normally on wild *P. nigra* foliage in August. After 2 d, mesh bags and caterpillars were removed, and one branch of each treatment was harvested. The remaining branches were harvested 10 or 29 d after the beginning of the experiment. On d 29, many of the trees were heavily infected with a rust fungus (*Melampsora*, sp.) on their leaves, which triggered early leaf fall and prevented some of the samples from being taken.

For sampling, leaves were excised and half of the leaf blade was cut along the midvein, wrapped in aluminium foil, and flash frozen in a Voyageur Plus transportable liquid nitrogen container (Air Liquide, Paris, France). Enclosed leaves were harvested separately from adjacent leaves growing between the original position of the mesh bag and the branch apex. After transport to the lab, samples were freeze-dried and transferred into 50 ml plastic tubes (Sarstedt, Nümbrecht, Germany). After adding approximately ten 4 mm steel beads, the leaf material was ground to fine powder by agitation on a paint shaker (Scandex, Pforzheim, Germany) and stored at −20 °C until further chemical analysis.

2010 Experiment– Effect of Herbivory on Phenolics in a Single Tree One male tree, which had large amounts of foliage accessible from the ground, was selected from the *P. nigra* population on Küstrin-Kietz Island. This tree was approximately 60 yr old, had a diameter of 100 cm at breast height, and was not investigated in the previous experiment in 2009. On July 12th 2010, a set of 60 experimental branches were chosen at a height <2 m. The newly developed apical shoots on each branch had about 20 fully developed leaves each and no side branches. In the herbivore treatment, ten 4th instar *L. dispar* were enclosed on the 6 basipetal leaves of each branch using the same mesh bags as in the previous experiment (Supplemental Fig. 1). To achieve maximum comparability among the branches in our experiment and reduce variation caused by ontogenetic differences, we always selected the six oldest leaves of each shoot as enclosed leaves since these were all produced in the first leaf flush at the beginning of the growing season. Mesh bags and caterpillars were removed after 40 h, and leaf and bark samples were taken for chemical analysis from 10 replicate branches 2, 4, and 7 d after caterpillar release. Controls were treated in the same way but caterpillars were not placed in the mesh bags. Branches of both treatments were randomly distributed over the lower crown. Experimental branches were well-spaced and never directly adjacent to minimize possible effects of long-distance defense signaling through the vascular system.

At each time point, ten randomly chosen branches per treatment were excised. The six most basal leaves (enclosed in the mesh bags) were sampled together without petiole and midvein. The bark of this basal section was excised with a scalpel. The same procedure was used to harvest the four leaves situated just apical to the mesh bag and their respective bark sections. All samples were promptly transferred into 5 ml plastic vials (Sarstedt, Nümbrecht, Germany) and flash-frozen. Tissue samples were processed as in the 2009 experiment (see above).

Identification of Phenolics in Black Poplar The phenolic compounds in the methanol extracts of leaf or bark tissue were analyzed by LC/MS. Separation was achieved on an Agilent 1100 Series LC system (Santa Clara, CA, USA) using a reversed phase column (EC 250/4.6 Nucleodur Sphinx, RP 5 μm, Macherey-Nagel, Düren, Germany) and a solvent system of 0.2 % aqueous formic acid and acetonitrile (ACN) employed at flow rate of 1 ml/min at a temperature of 25 °C. The proportion of ACN was increased from 14 % to 58 % in a linear gradient of 22 min. After the column was washed for 3 min with 100 % ACN, it was re-equilibrated to the initial eluent composition for 5 min prior to the next analysis. Mass spectra were recorded using an Esquire 6000 ESI-ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany). Phenolic compounds typically were analyzed in negative ionization mode with a skimmer voltage of 60 V, a capillary exit voltage of −121 V, and a capillary voltage of 4,000 V. Nitrogen was used as drying gas (11 ml/l, 330 °C) and nebulizer gas (pressure 35 psi).

Compounds were identified by mass spectra and chromatographic retention time in relation to standards. This procedure allowed the identification of all but one major and several minor peaks seen in the chromatogram recorded at 200 nm. The unidentified major peak had a UV spectrum similar to the salicinoids with $[M - H]^-=543$, and was postulated to be an OH-substituted derivative of the salicinoid tremulacin. To

acquire sufficient material for identification, a 200 g sample of *P. nigra* bark was extracted ×3 with approximately 400 ml MeOH. All extracts were combined, and the MeOH was evaporated to yield a brown, sticky residue that was treated with water and centrifuged. The supernatant was clarified with a 45 μl PTFE syringe filter (Roth, Karlsruhe, Germany) to yield a clear, orange solution that was freeze-dried. Approximately 1 g of the resulting white-orange solid was extracted with 1 ml MeOH to give an orange solution that was fractionated by semi-preparative HPLC using a reversed phase column (Supelcosil LC-18-DB semi-prep, 25 cm×10 mm×5 μm) eluted with a gradient of water and ACN. The ACN proportion was increased from 14 % to 35.3 % in 16 min followed by a 3 min wash (100 % ACN) and 3 min re-equilibration to the initial parameters. The major unidentified compound was collected in a peak eluting from 14.5 to 15.5 min. The combined fractions of approximately 100 injections of 5 μl each were then diluted with water and sorbed on a 1,000 mg RP-18 SPE cartridge (Merck, Darmstadt, Germany). The SPE cartridge was eluted with 1 ml of MeOH, and the MeOH removed from the eluate by evaporation to yield a white solid that was analyzed by NMR and identified as homaloside D by comparison with NMR data from the literature (Ekabo *et al.* 1993).

Assessing Experimental Herbivory In order to determine the extent of natural and experimental herbivory, we measured the missing leaf area of *L. dispar*-treated and control branches after harvesting on d 2 using the method of Mody and Linsenmair (2004). Briefly, leaves were arranged on a white board, covered with a Plexiglas panel to flatten the leaves, and placed next to a black 2×2 cm^2 reference square. This setup was photographed with a digital camera, and the missing leaf area was reconstructed using CS 5 Adobe Photoshop (Adobe Systems, San Jose, CA, USA). The pixel number of the missing leaf area was divided by the total (missing + present) leaf area and multiplied by 100 to calculate percent herbivory. The actual areas in cm^2 were calculated by comparison to the pixel number of the black 2×2 cm^2 square.

In the 2009 experiment, a subset of five randomly chosen enclosed leaves designated for the herbivory treatment were photographed and labeled *in situ* right before the caterpillars were released (day 0). The missing leaf area at this stage reflects the pre-experimental, natural herbivory. After caterpillar removal and leaf excision on day 2, the same leaves were photographed again to determine experimental herbivory.

In the 2010 experiment, the sampling method was modified to make it less time consuming. Ten branches were excised right before the experiment started, and the ten basal leaves (six enclosed and four adjacent) were removed and photographed. Additionally, the leaves from the control and herbivory treatment on d 2 were harvested and photographed. Since there was no statistical difference in damage between the leaves harvested on d 0 and the leaves from the control treatment of d 2 (data not shown), it was concluded that there was no significant activity of natural herbivores during the caterpillar treatment. Therefore, the level of herbivory of the leaves harvested on day 0 was not further considered.

Hormone Analysis A 20 mg portion of each sample was placed into the wells of a 96 well plate (Micronic, Lelystad, The Netherlands) and 1 ml MeOH containing 40 ng D_4-salicylic acid, 40 ng 9,10-D_2-9,10-dihydrojasmonic acid, 40 ng D_6-abscisic acid, and 8 ng JA-$^{13}C_6$-Ile as internal standards and one steel ball (3 mm diameter) were added. The mixture was extracted by agitation on a paint shaker (Scandex, Pforzheim, Germany) for 30 s and shaken for another 15 min at 200 rpm on a second shaker (IKA Labortechnik, Staufen, Germany). After centrifugation at 1, 000 g for 2 min, 700 μl of the supernatant were transferred into a new 96 well plate (Nunc, Roskilde, Denmark). The remaining sample was re-extracted twice more with 700 μl MeOH, centrifuged, and all three supernatants were combined to yield a pooled extract. A 500 μl aliquot of the pooled extract was mixed with an equal amount of water before analysis on an API 3200 LC/MS/MS system (Applied Biosystems, Carlsbad, CA, USA) using the chromatographic conditions and mass spectral parameters described in Vadassery *et al.* (2012). Concentrations of individual compounds were determined by comparing the response of each compound to that of its corresponding internal standard, multiplying by the amount of internal standard added and correcting for the sample mass.

HPLC Analysis The extraction of phenolics followed the protocol for hormone extraction, except that the MeOH in the first extraction step contained 0.8 mg/ml phenyl-β-glucopyranoside (Sigma Aldrich, St. Louis, MO, USA) as an internal standard instead of the isotopically-labeled hormones. Processed extracts were analyzed by HPLC/DAD (Agilent 1100 series, Santa Clara, CA, USA) using a solvent system of water and ACN but otherwise identical chromatographic conditions as described above for LC ESI ion-trap analysis.

Concentrations were calculated on the basis of the peak areas at 200 nm [internal standard, salicin, salicortin, proanthocyanidin B1 (PA B1)], 285 nm (homaloside D, (+)-catechin), and 330 nm (rutin, quercitrin) using standard curves prepared with pure substances. Samples for the standard curves also were spiked with the internal standard, and the analyte peak area-to-internal standard peak area ratio was used to calculate absolute amounts. Wavelengths were selected for each compound to optimize the sensitivity and linearity of the response. While salicin and PA B1 are typically quantified at 270–280 nm, the small amounts in our samples made quantification at 200 nm preferable. LC-MS analysis indicated no

other co-eluting substances. Due to the lack of pure standards available for quercitrin and PA B1, these compounds could not be quantified directly. Quercitrin was assumed to have an identical molar extinction coefficient as rutin since these compounds differ only in their sugar moieties, which are non-absorbing at 330 nm, and the concentration was calculated based on the standard curve of rutin. PA B1 was assumed to have a molar extinction twice that of catechin (at 200 nm) since it contains two catechin moieties.

Condensed Tannin Analysis Condensed tannins were analyzed by a variant of the BuOH:HCl method (Porter *et al.* 1986). A 5 mg portion of sample was weighed into a 15 ml plastic tube (Sarstedt, Nümbrecht, Germany). After addition of 5 ml digestion reagent (consisting of 2.78 mg $NH_4Fe(SO_4)_2$ * 12 H_2O, 270 µl 37 % HCl, and 130 µl water filled up to 5 ml with BuOH), the samples were heated in a water bath at 95 °C for 40 min. To account for interfering absorbing compounds, control samples were digested with butanol that was not amended with acid or iron sulfate. After cooling to room temperature, absorbances were read at 550 nm using a Spectronic 20 Genesys UV/VIS Photometer (Spectronic Instruments, Garforth, UK), and the absorbances of the control samples were subtracted from the absorbances of the analytical samples. Quantification was based on a calibration curve prepared with CT isolated from *Populus tremula* x *tremuloides* (Mellway and Constabel 2009). Low molecular weight flavan-3-ols (low MW flavan-3-ols) do not form colored anthocyanidins in the HCl:BuOH assay due to the lack of extender units (Schofield *et al.* 2001).

Statistical Analysis Phenolic and hormone data were analyzed with mixed-effects models using the lme package of the statistical software R 2.11.1 (R Development CoreTeam, http://www.R-project.org). If necessary, data were transformed (log, square root and reciprocal transformation) to meet the statistical assumptions. "Tree identity" (2009 experiment) or "branch replicate identity" (2010 experiment) entered the statistical model as random effects, and all data were nested within these. Starting from a constant null model the fixed effects were entered in the following sequence: "treatment" (control/herbivory), "branch section" (enclosed/adjacent), "sampling day", and the interactions "treatment x branch section" as well as "treatment x sampling day". "Sampling day" was excluded from the statistical model for hormones as these were not repeatedly measured. The *maximum likelihood* method was applied, and likelihood ratio tests were used to assess the statistical significance of model improvement. The *Akaike information criterion (AIC)* was used to determine the goodness of fit of the different models as well as to compare models. When no interaction effects were found, the models were simplified until the minimum adequate model was achieved (Crawley 2007). In the 2010 experiment, data for different "organ type" (leaves/bark) were analyzed separately with the same model as described above. Herbivory data were compared using an *ANOVA*. Throughout this manuscript, means always are displayed ± standard errors.

Results

Phenolic Composition of Black Poplar HPLC and LC/MS analysis of methanol extracts of bark tissue and foliage revealed the presence of four major classes of phenolics that were identified by UV and mass spectra and by chromatographic retention times in comparison to standards. Compounds identified included (1) salicinoids (salicin, nigracin, homaloside D, and salicortin), (2) flavan-3-ols (catechin, PA B1, and CT), (3) flavonol glycosides (rutin, quercitrin), and (4) simple phenolic acids (caffeic acid, ferulic acid, and 3,4-dimethoxy caffeic acid, trichocarpin) (Fig. 1). Salicinoids and CT were present in substantial amounts (10–150 mg g^{-1} DW) in both foliage and bark (Figs. 2, 3). Low molecular weight flavan-3-ols and flavonol glycosides were found in lower concentrations (2–10 mg g^{-1} DW), and flavonol glycosides were found only in foliage (Figs. 2, 3). For the amounts of the individual compounds, see supplemental material (Supplemental Tables 1, 2, 3).

Effects of Experimental Herbivory in 2009 Herbivory was imposed on branches of 20 trees by caging gypsy moth (*Lymantria dispar*) caterpillars on black poplar foliage for 2 days with mesh bags and phenolic compounds (Fig. 2, Table 1), and leaf areas were measured. After 2 days of feeding, the leaf area loss of herbivory enclosed leaves was 18.5±3.5 % compared to 3.3±0.7 % on control enclosed leaves.

Effects of Experimental Herbivory in 2010 In 2010, phenolics were measured in several samples from a single black poplar tree (Fig. 3, Table 2). Experimental herbivory inflicted for 2 days by gypsy moth caterpillars resulted in 22 % damage, while the adjacent unenclosed leaves showed only 12 % herbivory (Fig. 4, Table 3). On the control branches, the enclosed leaves showed only 5 % natural herbivory, while the adjacent unenclosed leaves had 12 % natural leaf area loss. These differences were significant for treatment ($P<0.01$) and the interaction of treatment x section ($P<0.01$). To determine if experimental herbivory induced defense-related signaling, we measured the levels of four hormones, salicylic acid (SA), jasmonic acid (JA), the jasmonic acid isoleucine conjugate (JA-Ile), and abscisic acid (ABA) in all leaves and bark sampled on day 2 (Fig. 4, Table 3). Herbivore treatment induced JA (~7-fold), JA-Ile (6-7-fold), and ABA (2.5-fold) (all $P<0.01$), but not SA, in the leaves. Increases were similar

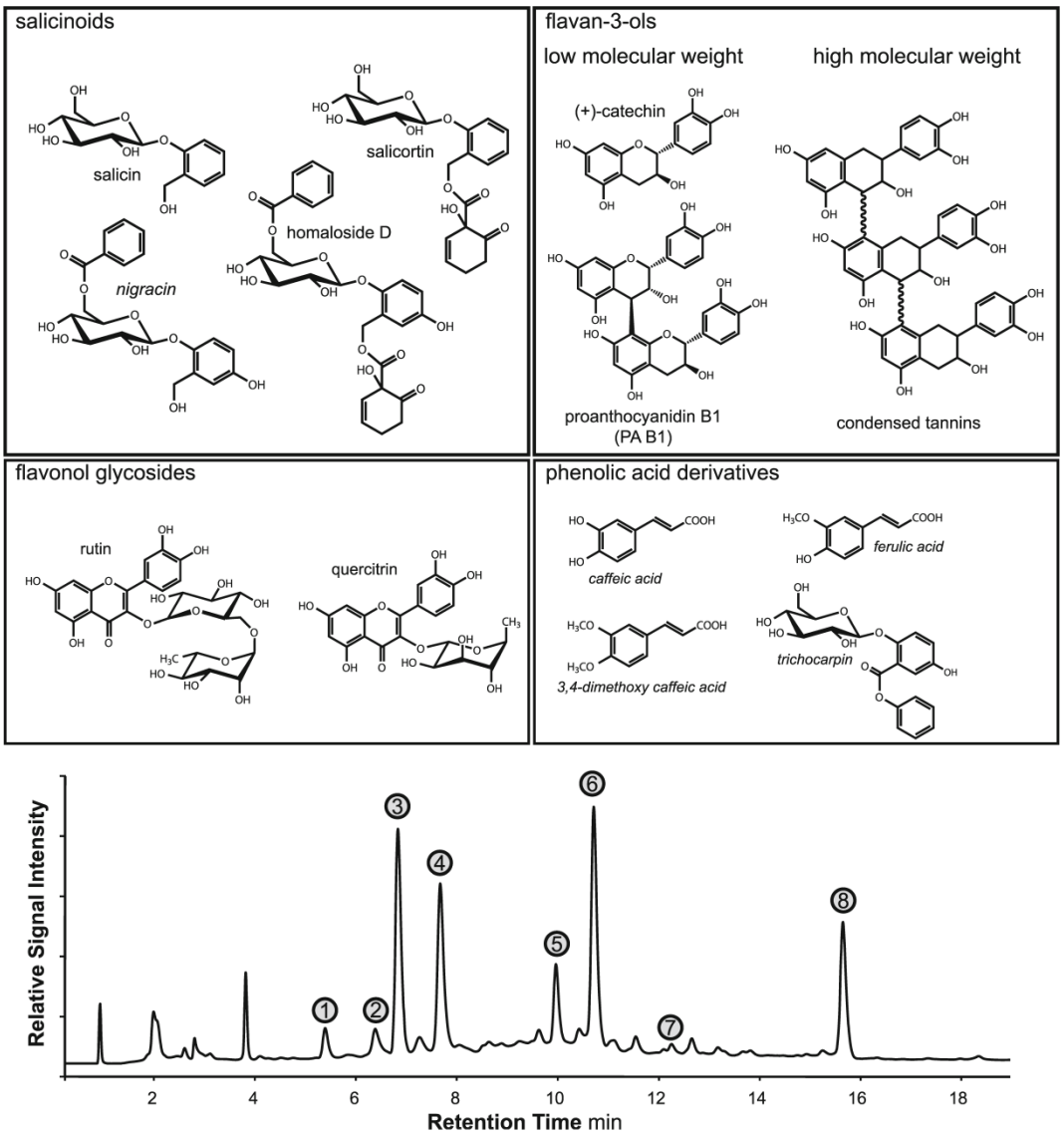

Fig. 1 Structures of phenolic compounds identified in *Populus nigra.* Compounds whose names are written in italics were not quantified. The HPLC/DAD-chromatogram shows a typical MeOH extract of *Populus nigra* leaves measured at 200 nm. *Numbers above peaks* indicate substance identity: **1** salicin; **2** proanthocyanidin B1; **3** phenyl-β-glucopyranoside (internal standard); **4** (+)-catechin; **5** rutin; **6** salicortin; **7** quercitrin; **8** homaloside D

in enclosed and adjacent leaves, except for JA which increased only ~2-fold in unenclosed leaves. In bark, patterns were more variable with significant decreases in ABA after experimental herbivory ($P<0.01$) in the bark of unenclosed branches. No significant effect of herbivory was observed on SA, JA, or JA-Ile in the bark.

2009 Experiment – Effect of Experimental Herbivory on Phenolics in 20 Trees Phenolic concentrations did not differ between herbivore-treated and control leaves 2 and 10 days after the beginning of herbivory, but by day 29 the salicinoid concentration in herbivore-treated leaves declined significantly ($P=0.04$) to about 40 % and 25 % of controls in enclosed and adjacent leaves, respectively (Table 1, Fig. 2).

Analysis of branches not treated with herbivores showed some changes in leaf phenolic concentration with leaf development when comparing the older, mesh bag-enclosed leaves (control enclosed) to the younger unenclosed leaves (control adjacent). Salicinoids were approximately half as concentrated in the older *vs.* the younger leaves ($P<0.01$). In contrast, older leaves contained about 50 % more low MW flavan-3-ols ($P<0.01$) and 20–30 % more CT ($P<0.01$) than the younger leaves. There were no significant differences in flavonol glycoside content ($P=0.36$).

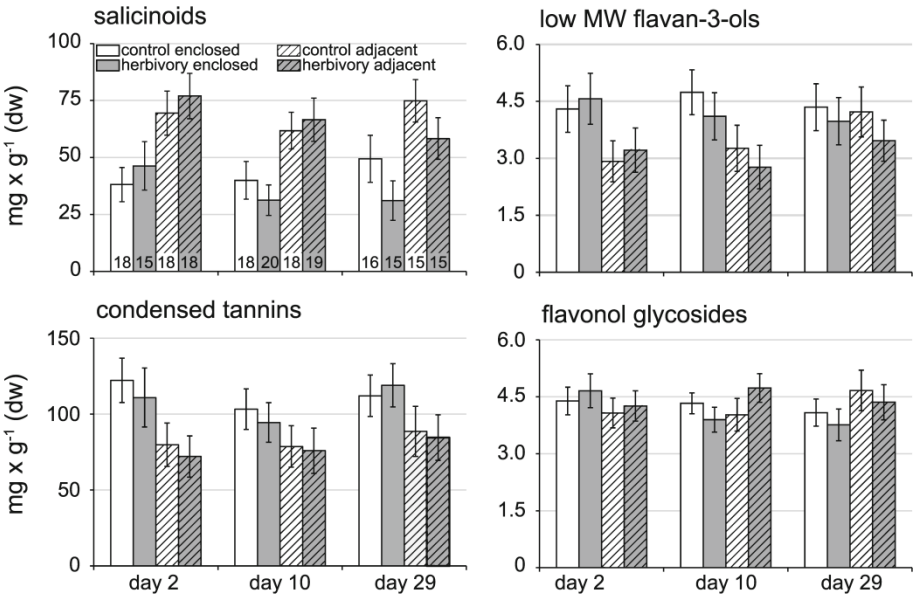

Fig. 2 Effect of gypsy moth (*Lymantria dispar*) herbivory on phenolic classes in black poplar (*Populus nigra*) in the 2009 experiment. Branches on 20 trees were enclosed and subject to feeding by gypsy moth larvae for 48 h or left as untreated controls. Younger leaves on the same branch adjacent to the enclosure were also analyzed for systemic effects of herbivory. Branches were sampled 2, 10 and 29 d after the beginning of the experimental herbivory. *Numbers at the bottom of the salicinoid columns* indicate the number of replicates which apply to all compound classes. *Error bars* show ± SE

2010 Experiment- Effect of Herbivory on Phenolics in a Single Tree Experimental herbivory had significant effects on the composition of phenolic compounds (Fig. 3, Table 2). The low MW flavan-3-ol concentration in damaged leaves decreased seven days after herbivory ($P=0.06$) by about 10 %, while the concentration in the bark increased ($P=0.04$) by about the same magnitude in the gypsy moth enclosures and in the adjacent unenclosed branch sections. Condensed tannins in the foliage increased in response to gypsy moth feeding ($P<0.01$) by not more than 20 % seven days after the onset of herbivory in the enclosures and the adjacent unenclosed leaves. There were no significant effects of experimental herbivory on the concentrations of salicinoids or flavonol glycosides.

Analysis of branches not treated with herbivores showed significant differences in phenolic composition between the older bark and foliage (which was enclosed) and the younger adjacent bark and foliage (which was left unenclosed). Salicinoid concentrations were much higher in younger than older leaves (about 5-fold, $P<0.01$), but less (up to 1/3 less, $P<0.01$) in younger *vs.* older bark sections. Low MW flavan-3-ols were significantly higher (up to 50 %, $P<0.01$) in older tissues, while condensed tannins were lower (by up to 1/3, $P<0.01$) in older bark, but were the same in the foliage. Over the seven day time course of the experiment, there were decreases (up to 25 %) in low MW flavan-3-ol content in leaves ($P<0.01$) and flavonol glycoside ($P=0.05$) content in leaves.

Discussion

The phenolics of the Salicaceae have been studied frequently for their role in anti-herbivore defense and their inducibility upon herbivore damage (Boeckler *et al.* 2011; Constabel and Lindroth 2010), but few studies have investigated large trees growing in natural stands. We found only sparse evidence for induction of phenolics in black poplar (*Populus nigra*) foliage and bark after leaf feeding by gypsy moth (*Lymantria dispar*) caterpillars. In the study conducted on 20 individual trees, no induction was observed, but rather a 20–40 % decrease in salicinoid concentration 4 weeks after damage. Low molecular weight flavan-3-ols, flavonol glycosides, and CT (condensed tannins) did not respond at all to gypsy moth feeding. A second experiment investigated the effect of gypsy moth herbivory on phenolics in the foliage and bark of single tree over a week. No induction of salicinoids or flavonol glycosides was observed. However, there were small but significant increases in low MW flavan-3-ols (catechin, proanthocyanidin B1) in the bark and CT in the foliage. The lack of greater response to herbivory was surprising in view of the large amount of leaf loss (22 % in gypsy moth-treated leaves *vs.* 5 % in control) and the 5- to 7-fold increase in jasmonate levels. Developmental stage and tissue type seem to be more important influences than herbivory on the phenolic content of old-growth *P. nigra* trees.

As the phytochemistry of *P. nigra* had not been investigated in detail, we characterized the most abundant phenolics of this tree. Compounds were classified into four groups (salicinoids, flavan-3-ols, flavonol glycosides, and phenolic acids, Fig. 1) according to their structures. Salicinoids, flavan-3-ols, and flavonol glycosides were quantified by HPLC or the BuOH:HCl assay, but the less abundant phenolic acids were not determined. Generally, the phenolic constitution resembles the profiles found in other Salicaceae (Donaldson *et al.* 2006; Förster *et al.* 2010; Heiska *et al.* 2007), but the salicinoid profile is species dependent. Of the salicinoids identified in

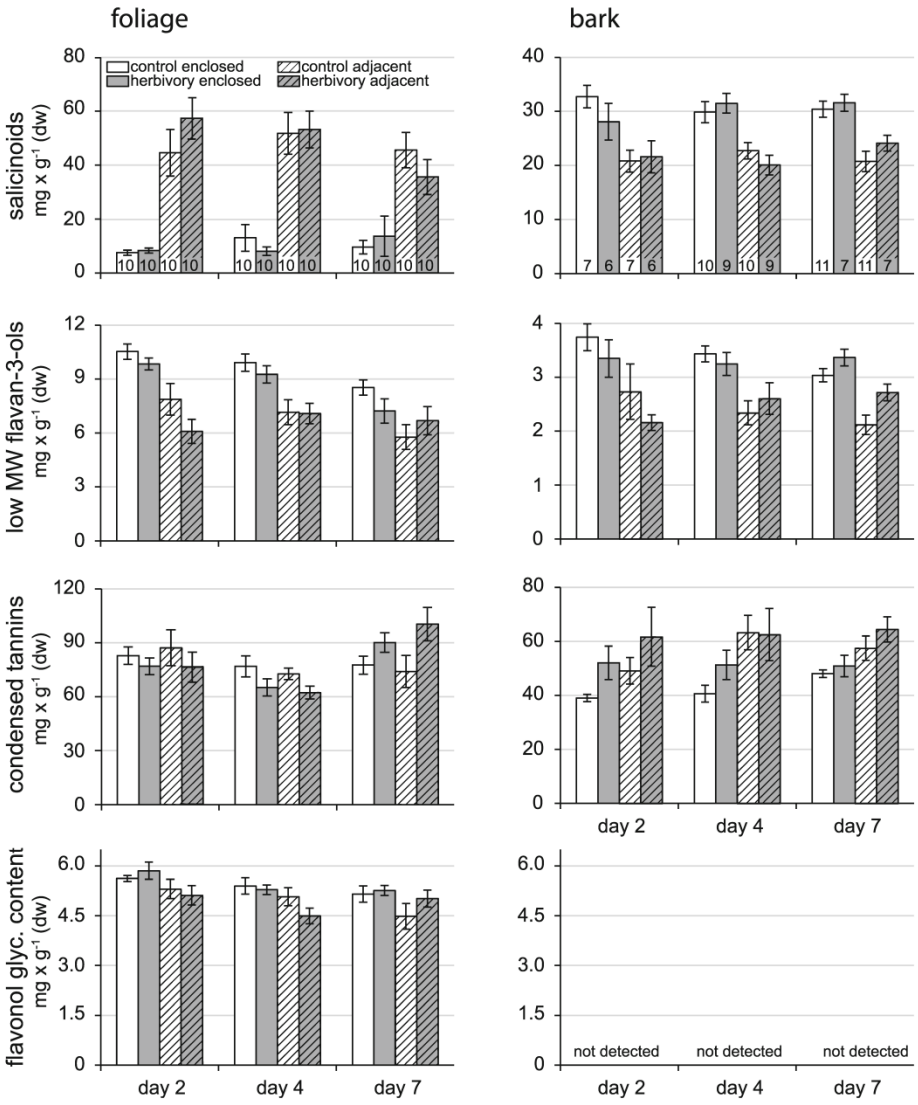

Fig. 3 Effect of gypsy moth herbivory on black poplar phenolic classes in the 2010. Experiment Branches on a single tree were enclosed and subject to 2 d of herbivory by gypsy moth larvae or left as uninfested controls. Younger bark and leaves on the same branch adjacent to the enclosure were also analyzed for systemic effects. Flavonol glycosides were not detected in the bark. *Error bars* show ± SE. Number of replicates are given at the column bottoms of the salicinoid graph and apply to all compound classes

black poplar, salicin and salicortin are widespread in the family, but nigracin and homaloside D are nearly restricted to this species. These two compounds contain a gentisyl alcohol substituent instead of the more common salicyl alcohol (Fig. 1). This type of salicinoid has been reported in other genera among the Salicaceae, such as *Homalium* (Ekabo *et al.*

Table 1 Results of the linear mixed effect model on changes of black poplar phenolics in response to herbivory treatment, branch section (older enclosed *vs.* younger unenclosed sections), and day of the time course in the 2009 experiment

	Treatment		Section		Day		Treatment × section		Treatment × day	
	F	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Salicinoids	1.17	0.28	58.00	**<0.01**	1.51	0.22	0.89	0.35	3.25	**0.04**
Low MW flavan-3-ols	1.09	0.30	24.00	**<0.01**	0.92	0.40	n.s.	n.s.	n.s.	n.s.
Condensed tannins	0.12	0.73	31.98	**<0.01**	2.76	0.07	n.s.	n.s.	n.s.	n.s.
Flavonol glycosides	0.03	0.86	0.83	0.36	<0.01	0.99	n.s.	n.s.	n.s.	n.s.

Bold numbers indicate significant effects, *n.s.* not significant

Table 2 Results of the mixed effect model on changes in the concentrations of black poplar phenolics in response to herbivory treatment, branch section (older enclosed *vs.* younger unenclosed sections), and day of the time course in the 2010 experiment

		Treatment		Section		Day		Treatment × section	
		F	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Salicinoids	leaves	<0.00	0.98	429.02	**<0.01**	0.77	0.48	n.s.	n.s.
	bark	<0.00	0.97	103.46	**<0.01**	0.07	0.93	n.s.	n.s.
Low MW flavan-3-ols	leaves	2.27	0.14	60.43	**<0.01**	5.74	**<0.01**	n.s.	n.s.
	bark	0.18	0.67	54.94	**<0.01**	0.93	0.40	3.12	**0.05**
Condensed tannins	leaves	0.00	>0.99	0.03	0.85	5.26	**<0.01**	5.36	**<0.01**
	bark	4.14	0.05	24.80	**<0.01**	1.44	0.25	n.s.	n.s.
Flavonol glycosides	leaves	0.00	0.98	22.27	**<0.01**	3.06	0.05	n.s.	n.s.
	bark	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

Bold numbers indicate significant effects, *n.s.* not significant, *n.d.* not determined

1993) or *Flacourtium* (Bourjot *et al.* 2012), but rarely in *Populus* or *Salix*. Like tremulacin and salicortin, homaloside D contains a 1-hydroxy-6-oxocyclohex-2-ene-1-carboxyl ester, a structural feature of salicinoids that increases toxicity to insect herbivores (Lindroth and Peterson 1988).

The use of herbivores to inflict experimental leaf damage is problematic due to the unpredictability of leaf area loss. This is especially true in the field because of possible variation among plants and the physical environment of the herbivore. In both experiments presented here, gypsy moth caterpillars consumed approximately 15 % of the leaf area offered within 2 days against a background of 5 % natural herbivory. Since plants "detect" herbivory in different ways (Hilker and Meiners 2010) evidence was sought to insure that the experimental damage treatment was substantial enough to evoke a response. Therefore, in our second experiment, the concentrations of the defense related hormones JA and JA-Ile were measured. These defense signaling molecules, which elicit anti-herbivore responses in many plant species (Erb *et al.* 2012), were elevated up to 7-fold by experimental herbivory in the 2010 experiment. Furthermore, a parallel field study that investigated the volatile emission of mature black poplar after gypsy moth feeding showed a strong release of volatiles under similar experimental conditions (Unsicker *et al.*, unpublished). The significant elevation in jasmonates indicated that the trees responded to the stimulus of experimental herbivory despite previous leaf damage, and could have been expected to show other defense responses.

Despite the elicitation of defense signaling, experimental gypsy moth feeding did not induce phenolic constituents of black poplar except for small increases in CT in the leaves and low molecular weight flavan-3-ols in the bark 7 days after herbivory (Fig. 3). Foliar induction of CT upon insect herbivore damage is a well-known phenomenon in young *Populus tremuloides,* and the time frame observed in our 2010 experiment is in agreement with studies on this species (Mellway *et al.* 2009; Peters and Constabel 2002; Stevens and Lindroth 2005). However, we observed only a weak increase of CT levels in the 2010 experiment and no significant changes in the 2009 experiment. Poplars increase their foliar CT levels with tree age (Donaldson *et al.* 2006), and it is conceivable that high constitutive levels limit a further induction in mature trees. Otherwise CT induction may not occur at all in black poplar, or is hampered by uncontrolled abiotic or biotic factors that inevitably occur in field studies.

In contrast to CTs and low MW flavan-3-ols, salicinoids usually are identified as the most important defenses against insect herbivores in the Salicaceae (Hemming and Lindroth 1995). Previous studies have sometimes shown salicinoid induction by herbivory, but declines or lack of response also have been reported occasionally (Julkunen-Tiitto *et al.* 1995; Osier and Lindroth 2001). Salicinoids were not induced in mature black poplar trees challenged by gypsy moth feeding. No changes in salicinoid concentration were detected 1 week after herbivore treatment (2010 experiment) and a decline was observed 4 weeks after treatment (2009 experiment). Similarly, Roth and coworkers (1998) observed significantly lower salicinoid levels in *P. tremuloides* after continuous 4 week forest tent caterpillar herbivory when compared to untreated controls, after a transient induction. Our experiments were conducted under natural field conditions, and hence treatment effects may have been masked by responses to other biotic and abiotic factors. The experiment in 2009 also was conducted in late summer when trees may invest fewer resources into defending deciduous leaves. Additionally, a fungal infestation by a *Melampsora* sp. (a typical late season occurrence in this population) led to premature leaf fall on practically all experimental trees. Therefore, decreasing levels of salicinoids with gypsy moth feeding could be due to accelerated senescence as a result of combined herbivory and fungal attack.

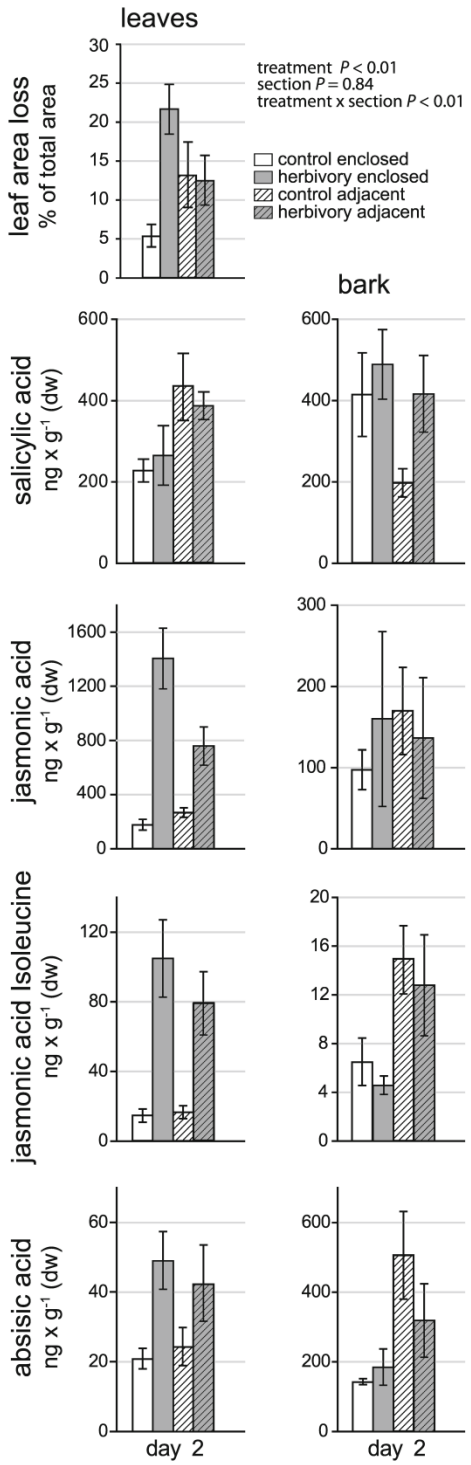

Fig. 4 Effect of gypsy moth herbivory on the leaf area and defense hormones in black poplar leaves and bark in the 2010 experiment All tissue was harvested immediately after the 48 h herbivory period. *P*-values refer to two-way ANOVA on the leaf area loss with treatment (herbivory or control) and section (enclosed or adjacent) as factors. *Error bars* show means ± SE. Number of replicates is identical with day 2 samples in Fig 2

Table 3 Results of the mixed effect model on changes in the concentrations of black poplar defense hormones in response to herbivory treatment and branch section (older enclosed *vs.* younger unenclosed sections) in the 2010 experiment

		Treatment		Section		Treatment × section	
		F	*P*	*F*	*P*	*F*	*P*
SA	leaves	0.09	0.77	13.22	**<0.01**	n.s.	n.s.
	bark	3.31	0.10	12.61	**<0.01**	3.85	0.08
JA	leaves	41.70	**<0.01**	0.00	0.96	22.14	**<0.01**
	bark	0.76	0.40	0.86	0.37	n.s.	n.s.
JA-Ile	leaves	31.72	**<0.01**	0.25	0.62	n.s.	n.s.
	bark	2.70	0.13	0.00	0.93	n.s.	n.s.
ABA	leaves	6.07	0.02	0.34	0.57	n.s.	n.s.
	bark	10.14	**<0.01**	1.70	0.22	n.s.	n.s.

Bold numbers indicate significant effects, *n.s.* not significant

Alternatively, the lack of salicinoid induction by herbivory in our study also could be due to the use of old-growth trees in our experiments, compared to most previous studies on poplar and willow, which investigated young trees or saplings. In contrast to young trees, old trees can store more nutrients in bark and roots. This storage may enable older trees to rely on compensatory growth as an alternative strategy to respond to herbivory (Haukioja and Koricheva 2000). Young trees with only a limited reserve of nutrients may rely more heavily on defense induction to counteract leaf area loss by herbivores.

Another explanation for the discrepancy with previous results may be that salicinoids are not induced in damaged leaves of black poplar, but only in young leaves produced after the defoliation event, as reported for *Populus tremuloides* (Stevens and Lindroth 2005). As we did not sample the new leaves produced during both experiments separately from existing leaves, we cannot exclude that salicinoid induction may have occurred in them.

The phenolic levels observed in both our experiments reflect ontogenetic patterns more than herbivore treatment. Comparison of samples from the older *vs.* younger leaves showed that salicinoid concentration was lower in old leaves, while the flavan-3-ol concentration was higher in old leaves. In bark samples, salicinoids and low molecular weight flavan-3-ol concentrations were higher in old samples, while CT was lower in old samples. The literature gives many examples of ontogenetic changes of phenolics in shoots of Salicaceae species, and declines in salicinoid levels with leaf age is a common pattern (Bingaman and Hart 1993; Kleiner *et al.* 2003; Meyer and Montgomery 1987; Young *et al.* 2010). It has been suggested that this decline may provide more anti-herbivore protection to younger tissue, which may be more valuable to the plant than older tissue and at a greater risk for attack due to its higher nutritional content (Bryant and Kuropat 1980).

In summary, the phenolic content was only modestly induced in old-growth black poplar trees growing in a natural stand after gypsy moth herbivory. Low MW flavan-3-ols and CT increased by 20 % or less, depending on the tissue, and salicinoids and flavonol glycosides did not increase at all. If phenolics serve in anti-herbivore defense, old trees may tolerate herbivory and regrow, rather than produce more chemical defenses. Alternatively, phenolics may be constitutively produced in levels adequate for defense.

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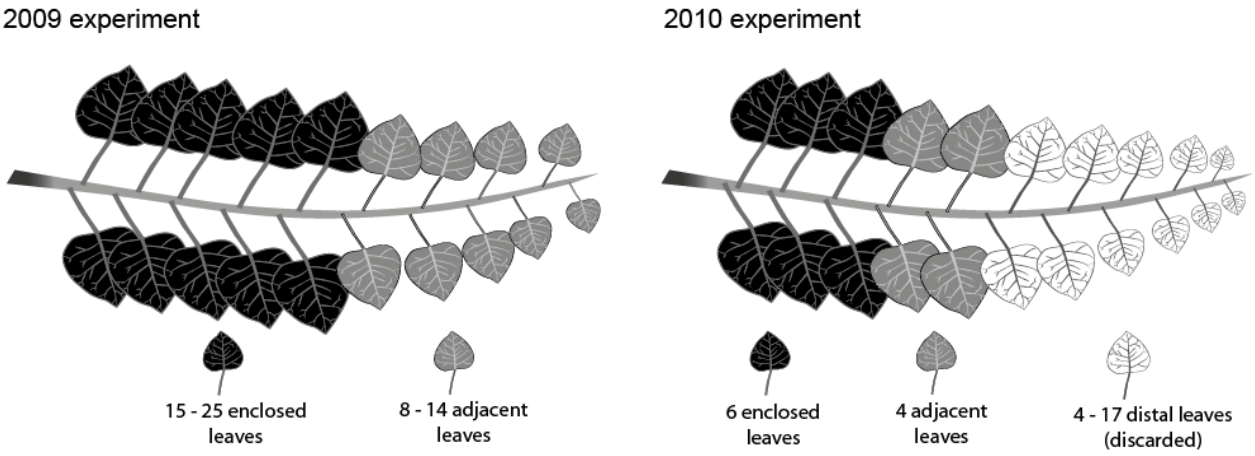
Appendix



Supplemental Fig. 1 Schematic illustration of the foliage sampled in the 2009 and 2010 experiment.

In 2009 20 different trees were investigated. Six branches of each tree were selected as experimental branches. In each of these branches a mesh bag was installed in a way that it enclosed a similar biomass of leaves (black leaves). Depending on the leaf morphology 10-25 branches were bagged. Some adjacent leaves between the bag and the branch apex were left unenclosed (grey leaves). In half of the mesh bags on each tree, five 4th-instar gypsy moth caterpillars were released. After 48 h, caterpillars and all mesh bags were removed, and one of branch of each treatment was excised and harvested. Enclosed leaves and adjacent leaves were sampled separately. The remaining four branches were sampled 10 and 29 d after the experiment had started (one of each treatment every time).

The 2010 experiment was conducted on one individual tree. In order to standardize the investigated tissue only fresh, non-woody shoots of this year were used as experimental branches (60 branches in total). The six basipetal leaves of each of the experimental branches were bagged and in half of the bags ten 4th instar caterpillars were released. After 2d, the caterpillars and all mesh bags were removed, and ten branches per treatment were harvested. From each branch, the six formerly encaged leaves as well as the four leaves situated just apical to the mesh bag (adjacent leaves) were sampled separately. The sampling procedure was repeated 4 and 7 d after the experiment had started.

Supplemental table 1. Concentration (mg/g dw) of individual phenolic compounds in the 2009 experiment. C=control, H=experimental herbivory.

enclosed bark

	day 2				day 4				day 7			
	C		H		C		H		C		H	
salicin	0.4 ± 0.1		0.4 ± 0.1		0.3 ± 0.0		0.4 ± 0.0		0.7 ± 0.1		0.6 ± 0.1	
salicortin	15.6 ± 0.7		14.3 ± 1.4		14.3 ± 0.8		14.8 ± 0.8		14.6 ± 0.5		15.0 ± 0.5	
homaloside D	16.7 ± 1.6		13.4 ± 2.2		15.2 ± 1.4		16.3 ± 1.2		15.1 ± 1.2		16.0 ± 1.2	
(+)-catechin	3.0 ± 0.2		2.7 ± 0.2		2.8 ± 0.1		2.6 ± 0.2		2.5 ± 0.1		2.7 ± 0.1	
PA B1	0.7 ± 0.0		0.7 ± 0.1		0.6 ± 0.0		0.6 ± 0.1		0.6 ± 0.0		0.6 ± 0.0	
quercitrin	n.d. ± n.d.		n.d. ± n.d.		n.d. ± n.d.		n.d. ± n.d.		n.d. ± n.d.		n.d. ± n.d.	
rutin	n.d. ± n.d.		n.d. ± n.d.		n.d. ± n.d.		n.d. ± n.d.		n.d. ± n.d.		n.d. ± n.d.	
CT	39.0 ± 1.3		52.0 ± 6.2		40.6 ± 3.1		51.3 ± 5.4		48.0 ± 1.4		50.9 ± 4.0	

adjacent bark

	day 2				day 4				day 7			
	C		H		C		H		C		H	
salicin	0.3 ± 0.0		0.3 ± 0.0		0.3 ± 0.1		0.4 ± 0.1		0.6 ± 0.1		0.3 ± 0.0	
salicortin	12.3 ± 0.7		12.7 ± 1.2		13.0 ± 0.5		12.0 ± 0.6		12.0 ± 0.8		14.1 ± 0.7	
homaloside D	8.2 ± 1.4		8.7 ± 1.8		9.4 ± 1.1		7.7 ± 1.4		8.1 ± 1.1		9.7 ± 0.9	
(+)-catechin	2.2 ± 0.5		1.7 ± 0.1		1.8 ± 0.2		2.0 ± 0.2		1.6 ± 0.2		2.1 ± 0.1	
PA B1	0.5 ± 0.0		0.5 ± 0.0		0.5 ± 0.0		0.6 ± 0.1		0.5 ± 0.0		0.6 ± 0.0	
quercitrin	n.d. ± n.d.		n.d. ± n.d.		n.d. ± n.d.		n.d. ± n.d.		n.d. ± n.d.		n.d. ± n.d.	
rutin	n.d. ± n.d.		n.d. ± n.d.		n.d. ± n.d.		n.d. ± n.d.		n.d. ± n.d.		n.d. ± n.d.	
CT	49.1 ± 4.9		61.7 ± 10.9		63.3 ± 6.3		62.5 ± 9.6		57.5 ± 4.5		64.4 ± 4.6	

Supplemental table 2. Concentration (mg/g dw) of individual compounds in the 2010 experiment in leaves. C=control, H=experimental herbivory.

enclosed leaves

	day 2		day 4		day 7	
	C	H	C	H	C	H
salicin	0.9 ± 0.1	1.0 ± 0.1	1.1 ± 0.3	0.9 ± 0.1	1.1 ± 0.2	1.0 ± 0.2
salicortin	6.1 ± 0.7	6.9 ± 0.6	9.5 ± 3.1	6.2 ± 1.0	7.4 ± 1.6	9.8 ± 4.6
homaloside D	0.6 ± 0.2	0.5 ± 0.2	2.4 ± 1.5	1.1 ± 0.5	1.1 ± 0.7	2.8 ± 2.6
(+)-catechin	8.0 ± 0.3	7.5 ± 0.2	7.5 ± 0.4	7.1 ± 0.3	6.7 ± 0.3	5.7 ± 0.5
PA B1	2.5 ± 0.1	2.3 ± 0.1	2.4 ± 0.1	2.1 ± 0.1	1.9 ± 0.1	1.6 ± 0.2
quercitrin	0.9 ± 0.1	1.1 ± 0.1	1.0 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	0.9 ± 0.1
rutin	4.7 ± 0.1	4.8 ± 0.2	4.4 ± 0.2	4.5 ± 0.1	4.3 ± 0.2	4.4 ± 0.1
CT	82.9 ± 4.8	39.0 ± 1.3	76.9 ± 5.8	40.6 ± 3.1	77.6 ± 5.0	48.0 ± 1.4

adjacent leaves

	day 2		day 4		day 7	
	C	H	C	H	C	H
salicin	2.4 ± 0.5	3.0 ± 0.3	1.9 ± 0.2	2.5 ± 0.2	3.3 ± 0.4	3.0 ± 0.5
salicortin	29.2 ± 5.0	36.7 ± 4.5	34.0 ± 4.5	33.5 ± 3.8	29.3 ± 4.1	23.7 ± 4.1
homaloside D	13.0 ± 3.3	17.6 ± 2.9	15.9 ± 3.2	17.2 ± 3.0	13.0 ± 2.1	9.0 ± 2.1
(+)-catechin	6.3 ± 0.7	4.9 ± 0.5	5.6 ± 0.5	5.6 ± 0.4	4.7 ± 0.5	5.5 ± 0.6
PA B1	1.6 ± 0.2	1.2 ± 0.2	1.5 ± 0.2	1.5 ± 0.1	1.1 ± 0.2	1.2 ± 0.1
quercitrin	1.2 ± 0.1	1.2 ± 0.1	1.1 ± 0.1	1.0 ± 0.0	1.0 ± 0.1	1.2 ± 0.1
rutin	4.1 ± 0.2	3.9 ± 0.2	4.0 ± 0.2	3.5 ± 0.2	3.5 ± 0.3	3.8 ± 0.2
CT	87.3 ± 10.0	76.5 ± 8.3	72.7 ± 3.1	62.4 ± 3.5	74.0 ± 9.0	100.4 ± 9.3

Supplemental table 3. Concentration (mg/g dw) of individual compounds in the 2010 experiment in bark. C=control, H=experimental herbivory.

enclosed bark

	day 2				day 4				day 7			
	C		H		C		H		C		H	
salicin	0.4 ± 0.1		0.4 ± 0.1		0.3 ± 0.0		0.4 ± 0.0		0.7 ± 0.1		0.6 ± 0.1	
salicortin	15.6 ± 0.7		14.3 ± 1.4		14.3 ± 0.8		14.8 ± 0.8		14.6 ± 0.5		15.0 ± 0.5	
homaloside D	16.7 ± 1.6		13.4 ± 2.2		15.2 ± 1.4		16.3 ± 1.2		15.1 ± 1.2		16.0 ± 1.2	
(+)-catechin	3.0 ± 0.2		2.7 ± 0.2		2.8 ± 0.1		2.6 ± 0.2		2.5 ± 0.1		2.7 ± 0.1	
PA B1	0.7 ± 0.0		0.7 ± 0.1		0.6 ± 0.0		0.6 ± 0.1		0.6 ± 0.0		0.6 ± 0.0	
quercitrin	n.d. ± n.d.		n.d. ± n.d.		n.d. ± n.d.		n.d. ± n.d.		n.d. ± n.d.		n.d. ± n.d.	
rutin	n.d. ± n.d.		n.d. ± n.d.		n.d. ± n.d.		n.d. ± n.d.		n.d. ± n.d.		n.d. ± n.d.	
CT	39.0 ± 1.3		52.0 ± 6.2		40.6 ± 3.1		51.3 ± 5.4		48.0 ± 1.4		50.9 ± 4.0	

adjacent bark

	day 2				day 4				day 7			
	C		H		C		H		C		H	
salicin	0.3 ± 0.0		0.3 ± 0.0		0.3 ± 0.1		0.4 ± 0.1		0.6 ± 0.1		0.3 ± 0.0	
salicortin	12.3 ± 0.7		12.7 ± 1.2		13.0 ± 0.5		12.0 ± 0.6		12.0 ± 0.8		14.1 ± 0.7	
homaloside D	8.2 ± 1.4		8.7 ± 1.8		9.4 ± 1.1		7.7 ± 1.4		8.1 ± 1.1		9.7 ± 0.9	
(+)-catechin	2.2 ± 0.5		1.7 ± 0.1		1.8 ± 0.2		2.0 ± 0.2		1.6 ± 0.2		2.1 ± 0.1	
PA B1	0.5 ± 0.0		0.5 ± 0.0		0.5 ± 0.0		0.6 ± 0.1		0.5 ± 0.0		0.6 ± 0.0	
quercitrin	n.d. ± n.d.		n.d. ± n.d.		n.d. ± n.d.		n.d. ± n.d.		n.d. ± n.d.		n.d. ± n.d.	
rutin	n.d. ± n.d.		n.d. ± n.d.		n.d. ± n.d.		n.d. ± n.d.		n.d. ± n.d.		n.d. ± n.d.	
CT	49.1 ± 4.9		61.7 ± 10.9		63.3 ± 6.3		62.5 ± 9.6		57.5 ± 4.5		64.4 ± 4.6	

3.5. Manuscript V: Spatiotemporal heterogeneity of phenolics in wild black poplar (*Populus nigra*)

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Abstract

Plant secondary metabolites are not homogenously distributed within plants and additionally subject to temporal variation. In the temperate zones, temporal variation is often related to seasonal factors, while intra-plant variation is often explained with the "value" of a plant organ or its developmental state. As many secondary metabolites are involved in the defense against pathogens or herbivorous animals, both, the temporal and spatial heterogeneity may have a considerable influence on higher trophic levels. However, annual trajectories and distributions of secondary metabolites are often compound-specific and only few species have been thoroughly investigated. In this manuscript we present an annual recording of the phytochemistry in 20 wild black poplar trees together with data on the spatial heterogeneity of phenolic compounds. We found that the two major phenolic classes in black poplar, salicinoids and condensed tannins, increase in the course of the vegetation period, while phenolic acids, flavonol glycosides and nitrogen declined. We also document that salicinoids show higher concentrations in the lower crown part, in addition to the well-known increase along the shoot axis. Recordings of leaf damage by chewing herbivores revealed that levels occurred early in the year and in tissues with high levels salicinoid levels. As these phenolics are known to be potent herbivore defenses, our results suggest that black poplar configures its spatial salicinoid distribution according to the optimal defense theory. However, a correlative approach showed that salicinoids do not affect the amount of damage inflicted by chewing herbivores. Instead, we found that catechin may have fungicidal activity, since this compound was negatively correlated to fungal infestation.

Introduction

Plants produce secondary metabolites that are adverse to herbivores. Compounds that are immediately negative to herbivores (e.g. toxic or deterrent) are commonly called direct defenses. Evidence for direct defense has been found in virtually all model plant species and attributed to specific compound classes, for example glucosinolates in *Arabidopsis thaliana* (Halkier & Gershenzon 2006), benzoxazinoids in *Zea mays* (Maag *et al.* 2014) or cyanogenic glycosides in *Sorghum* (Zagrobelny *et al.* 2004). Interestingly, the levels of these direct defense compounds show considerable patterns of variation. Hence, the adverse effect on potential herbivores is variable. As this variation in secondary metabolism follows patterns, such as seasonal development, it is partially predictable and allows herbivores to adapt and avoid high levels of defense. In turn, plants may adapt the levels of direct defense to the occurrence of herbivores leading to a complex interaction. The plant intrinsic and environmental factors leading to temporal and spatial variation will be shortly addressed in the following paragraphs.

In many climate zones, plants cycle annually through unfavorable conditions and therefore many perennial species maintain a deciduous life style. In such plants, the availability of photosynthates is restricted to the vegetation period and the metabolism is subjected to annual fluctuations. During the vegetation period the carbon for reproduction, growth and secondary metabolism has to be fixed but also the resources for the next year's foliage have to be allocated and stored. This scenario is widely believed to be a dilemma, because resources are limiting and strengthening one process is a trade-off at the cost of another (i.e. grow or defend). At the beginning of the vegetation period, when biomass has to be generated but photosynthesis is still low, allocation to defense compounds should be limited. On the other hand, young tissue is considered especially valuable and often better defended than mature plant parts (McKey 1974). In oak, phytochemical analysis has shown that condensed tannins increase in spring, while the levels of hydrolysable tannins decline (Mauffette & Oechel 1989; Salminen *et al.* 2004). The initially low levels of condensed tannins and the rapid early year decline of nitrogen were formerly believed to be drivers of food quality for herbivores and were held responsible for the high herbivore densities typically observed in spring (Feeny 1970). However, later studies with oak and birch challenged a direct relationship between single phytochemical traits and herbivore performance (Haukioja, Ossipov & Lempa 2002) and other tree species besides representatives of *Quercus* and *Betula* have been rarely investigated with respect to the seasonality of phytochemistry and herbivore occurrence.

In addition to seasonal changes, phytochemistry can vary spatially within a plant and thus some plant parts may be more attractive to herbivores than others. This phenomenon is often related

to ontogeny. As mentioned above, the levels of secondary metabolites and nutrients are often higher in the juvenile state of a plant organ and decline during maturation. Such innate patterns were observed in herbaceous plant species (Brown *et al.* 2003), and woody perennials (Tahvanainen *et al.* 1985) alike. In poplar ontogenetic changes occur within shoots (Kleiner *et al.* 2003; Rehill *et al.* 2006) and on the whole tree level (Donaldson *et al.* 2006), so that herbivores will encounter different food qualities in young and old trees and when feeding on young and old leaves. Plant traits are also known to adapt locally to given environmental conditions, a process called phenotypic plasticity. A special and extensively studied case of phenotypic plasticity is the induction of defenses, i.e. increasing amounts of direct defense compounds in response to herbivory (Zangerl 2003). However, the impact of other environmental influences, such as changing climatic parameters with height or direction, has received little attention so far.

In the present study we aimed to elucidate the seasonal and spatial dynamics of phenolic secondary metabolites and nutrients in black poplar (*Populus nigra*) in an attempt to link these parameters to insect herbivory and pathogen infestation. Black poplar, a typical flood-plain species endemic to Europe, appears well-suited for such an approach as it produces high amounts of various phenolics. The major phenolics of this species have been described previously and the foliage contains high amounts of salicinoids in addition to condensed tannins (Boeckler, Gershenzon & Unsicker 2013). The former were the focus of this study due to their function in herbivore defenses (Boeckler, Gershenzon & Unsicker 2011) while condensed tannins and related flavan-3-ols were interesting because of their putative role as anti-pathogen defenses (Miranda *et al.* 2007). We also analyzed small phenolics of minor abundance and nitrogen in order to get a more complete estimate of the food quality of black poplar foliage for herbivores and correlated leaf chemistry to measures of herbivore infestation (leaf area loss) and fungal load (quantification of foliar chitin). Experiments were conducted in the field with 10 female and 10 male trees of a natural black poplar population to address the following questions: 1) What is the trajectory of phenolic contents in black poplar foliage over the course of the vegetation period? 2) Is there a spatial heterogeneity of phenolics within the tree? 3) How is the spatiotemporal variation linked to the occurrence of herbivores? 4) Do phenolics confer protection against herbivores or pathogens?

Material and Methods

Study site and tree individuals

The trees investigated in this study were representatives of a natural population of black poplar (*Populus nigra*) located in a floodplain forest of the river Oder in north-east Germany (52°34'1''N, 14°38'3''E). Temperature and precipitation measurements in 2009-2010 recorded an average of 11.5 °C and 402.3 mm, respectively. The black poplar population consists of about 350 individuals that are scattered over an area of approximately 1.5 km^2. Sampling was conducted on ten male and ten female trees that were selected along a 750 m long north-east to south-west transect. Experimental trees were all mature individuals, approximately 20-30 m high and 30-60 years old.

Sampling of plant material

Three replicate branches from experimental each tree were sampled nine times within 14 months (2010: February 4th, April 7th, May 1st, June 15th, July 13th, August 23rd, October 8th; 2011: February 2nd, April 15th) in approximately 7 m height with a long-reach pruner. From April 7th to June 15th 2010 an additional three replicate branches were harvested from 1 m height. Bark was sampled from a 5 cm long section of the main branch starting from the 4th side branch in an apical direction (see supplemental for scheme). Developmental processes in this part of the branch were expected to be minor and any variation seasonal rather than developmental. Foliage was harvested during the vegetation period between May 1st and August 23rd 2010. The first leaf sampling was conducted approximately two weeks after bud break when the foliage was still fresh and soft. The first 25 leaves counted from the branch apex downwards were sampled. At the later time points, only the first 10 leaves were sampled. On October 8th many trees had already shed their leaves as a consequence of leaf rust infestation (*Melampsora* spp.) and no sampling was possible. Leaf blades were removed from the midvein and sampled separately from petioles. On April 15th 2011 many trees were flowering and flowers from experimental branches were also sampled.

In 2012 vertical patterns of phenolic distribution were investigated within two trees. Ropes were installed within these two trees to permit sampling from different heights. Both trees were sampled three times during the vegetation period in approximately four week intervals (May 16th, June 26th and August 2nd). At each date, five replicates of the apical part of a branch from four different heights (between 1-18 m) were excised. Each leaf (laminae without petiole) of the main shoot was sampled separately together with the main shoot and the bark of the growth

of last year (see supplemental). Transitions between wood of different years have a crinkled bark texture and can be easily recognized.

Herbivory measures

Present and missing leaf area was determined as described in Boeckler, Gershenzon and Unsicker (2013). Briefly, all leaves of a specific branch were photographed together and the missing leaf area was reconstructed using Adobe Photoshop. The reconstructed leaf area was divided by the total leaf area (missing + present) and multiplied by 100 to calculate % herbivory. Only herbivory that caused a total loss of all tissues along the leaf cross axis, e.g. herbivory by larger chewing herbivores was included. Superficial or skeletonizing damage, as caused by small herbivores, or leaf mining could not be analyzed with the method applied. For example damage by leaf beetle larvae (*Phratora* sp), was not included.

Sample conservation and processing

During the dormancy period, abscised branches were stuck into wet sand, transported to the lab and stored at 4 °C until sampling. Samples were processed within 48 h after abscission. Metabolic processes were expected to be limited due to the low temperatures and water content and the impact of sampling procedure on phenolic contents was expected to be minimal. Sampled material was transferred into 5 ml plastic tubes, flash-frozen in liquid nitrogen and stored at -20 °C until freeze-drying. The freeze-dried material was ground to a fine powder by agitation on a paint shaker (Scandex, Pforzheim, Germany) after addition of approximately 10 steel beads (diameter 4 mm). Powdered samples were stored at -20 °C until chemical analysis. During the vegetation period, abscised branches were stuck into water until sampling within the next 3 h. All tissues were sampled on-site, wrapped into aluminum foil and flash frozen in a transportable liquid nitrogen container (Voyageur Plus, Air liquid). After transport to the lab, samples were freeze-dried and processed as described above.

Quantification of phenolics

Throughout this manuscript, phenolics are summarized in biosynthetic subclasses (Mellway & Constabel 2009), namely salicinoids (also termed phenolics glycosides or salicylates), catechins, condensed tannins, flavonol glycosides, and phenolic acids. We adopted this procedure for simplicity, although some subclasses are represented by only two compounds (compounds and their structures are shown in supplemental). Phenolic compounds were quantified as described in Boeckler, Gershenzon and Unsicker (2013). Briefly, methanol

extracts of powdered samples were analyzed by HPLC to quantify small phenolic compounds. Condensed tannins were determined by a variant of the Porter assay (Porter, Hrstich & Chan 1986). For quantitative assessment of fungal infection, the concentration of chitin, an N-acetylglucosamine polymer, which is a component of fungal cell walls, was determined. The polymer was chemically digested to form glucosamine (Zhu *et al.* 2005), which was derivatized with FMOC to ensure good chromatographic separation from other mono-saccharides. The detailed procedure is described in the supplemental material. The FMOC-derivative was quantified by LC/MS using mannosamine as internal standard.

Statistical analysis

Data of both experiments were aggregated using SPSS to avoid pseudo-replication. In the 2010 experiment all data were averaged over the three replicates per tree and date. In the 2012 experiment compounds analyzed by HPLC were averaged over all leaves of a branch to compare the different heights. In turn, all heights were aggregated when the effect of leaf age was analyzed. Note that concentrations are strictly regarded as proportion data and averaging them, for example in all leaves of one branch, will not necessarily reflect the concentration of the bulk sample of all leaves, because the contribution of the leaf mass to the overall sample mass is ignored. Nevertheless, we still prefer this method to give more emphasis to developing leaves which have low weights.

Statistical analysis was conducted with R 2.15.0. We used linear mixed effect models ("lme" function) that are widely applied for analyzing data with temporal and spatial pseudoreplication (Crawley 2007). In all models the fixed effects were tested against a Null model (~1) and the maximum likelihood method was applied. Significance was tested by model comparison using the anova function. Data were square root- or log-transformed if necessary. In the 2010 experiment sampling date and tree identity entered the model as random effects. Date (as factor), sex and their interaction were added as fixed effects.

In the 2012 experiment, both trees were analyzed separately due to the large genotypic differences in phenolic contents. Two statistical analyses were carried out to elucidate the effect of height and leaf position within a shoot. To analyze the former, the phenolic levels of all leaves within a branch were averaged, so that each of the five branches harvested per height was represented by one value, and nested within date (random effect). Date (as factor), height and their interaction entered the model as fixed effects. For the analysis of the phenolic distribution along the shoot axis, leaves were assigned to three pools depending on their position: The three basal and the three apical leaves represented the "old" and the "young" leaf

pool, respectively. The influences of date and age class (fixed effects) on phenolics were tested using date and branch replicates as random factors.

Results

Seasonal variation in phytochemistry and herbivory

Leaf blades contained all compounds (3 salicinoids, 2 catechins, 2 flavonol glycosides, 4 phenolic acids condensed tannins) analyzed and specific trajectories over the vegetation period were found (**Fig. 1**). Salicinoid levels increased about 4-fold during summer ($p=0.001$) and showed the strongest increase between June and July. Catechins were undetectable in May, peaked in June and decreased by roughly 50 % thereafter ($p=0.007$). Flavonol glycosides levels decreased to one third of the early year concentration ($p<0.001$). Large amounts of phenolic acids were only found in spring followed by a dramatic decrease ($p<0.001$). By contrast, condensed tannin levels were low in May but increased 4-fold in June and remained constant until August ($p=0.001$). Besides the season, some phenolic were also significantly influenced by sex. Female trees produced significantly more salicinoids but less CT than the male trees ($p=0.048$ and $p<0.0001$, respectively). Herbivory was significantly influenced by the sampling date ($p=0.008$). Leaf damage was very low after leaf flush and increased 5-fold in the following six weeks. Between June and August a significant decline was observed, potentially caused by the shed of severely damaged leaves. Carbon and nitrogen were also subjected to seasonal patterns. Nitrogen concentrations dropped significantly ($p<0.001$) over time due to a 50 % decline in the first 1.5 month after leaf flush. Carbon levels declined only slightly but in a sex-dependent manner (date x sex $p=0.042$) and female trees contained significantly more carbon in the first half of the vegetation period (sex, $p=0.015$).

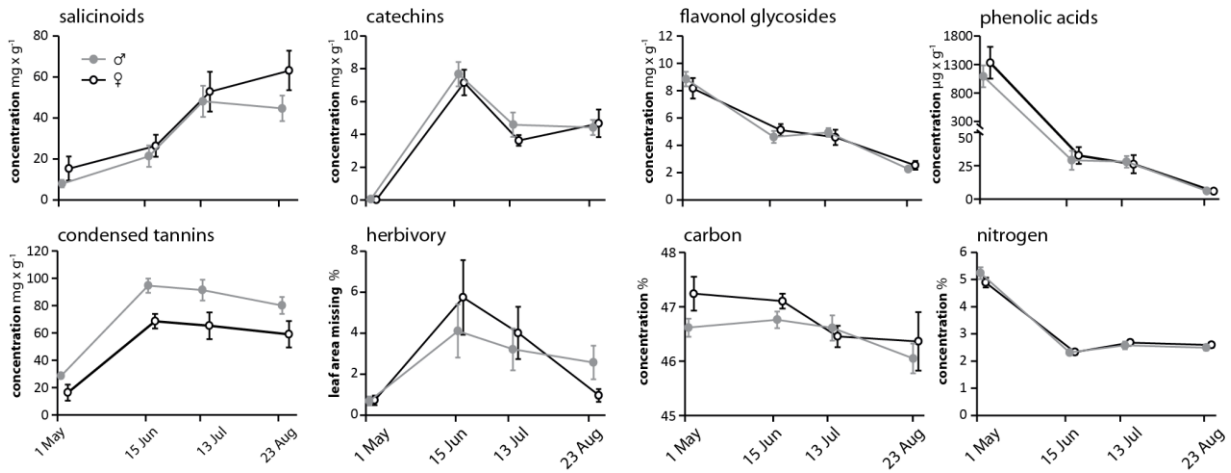

Fig. 1. Seasonal trajectories of phenolics, herbivory, nitrogen and carbon of *Populus nigra* foliage. Each data point represents the mean ± SE of 10 individual trees. Means of single trees were calculated from one (herbivory, carbon, nitrogen) or three (phenolics) replicates per date, respectively.

Table 1. Results of linear mixed effect model on seasonal variation of phenolics in male and female *Populus nigra* trees. Corresponding data are shown in Fig. 1.

	salicinoids		catechins		flavonol glyc.		phen. acids	
	p	AIC	*p*	AIC	*p*	AIC	*p*	AIC
date	0.001	178.7	0.007	268.2	<0.001	43.2	<0.001	230.5
sex	0.048	176.8	0.434	269.6	0.898	45.2	0.590	232.2
date x sex	0.828	181.9	0.635	272.7	0.470	48.7	0.666	236.7

	CT		herbivory		carbon		nitrogen	
	p	AIC	*p*	AIC	*p*	AIC	*p*	AIC
date	0.001	702.9	0.008	262.8	0.190	160.3	<0.001	98.8
sex	<0.001	686.5	0.518	264.4	0.015	156.4	0.772	-96.8
date x sex	0.105	686.4	0.848	269.6	0.042	154.1	0.367	-94.0

Stratification of phytochemistry and herbivory

Foliage samples from different heights revealed that phenolic compounds are not evenly distributed over the tree crown. Both, the 2010 and 2012 experiment documented that salicinoids are more abundant in foliage near the ground and that this pattern persists over time (**Fig. 2, Table 2**). In 2012 salicinoid concentrations measured from 5m and above were similar, indicating that the elevated levels are restricted to ground near foliage (**Fig. 2, Table 2**). Catechins were also significantly influenced by height, but no robust pattern was observed. Significant differences were found in June 2010 but this was not reproducible in 2012 (**Fig. 2**). Phenolic acids and condensed tannins were found to be significantly lower at ground level in 2010, but this pattern was not stable over time (height x date interaction significant). Herbivory levels in 2010 were significantly higher in June than in April and overall slightly higher in the foliage of the lower canopy. In 2012 the herbivory data lacked sufficient replication within heights and was too variable to be analyzed.

Distribution of phenolic compounds along the shoot axis

The distribution of phenolics along the shoot axis was determined on the single leaf level, but data were pooled to facilitate the statistical analysis and display of results. Salicinoids increased along the shoot axis and were roughly 2-fold higher in the young leaves compared to the old leaves (age class: $p<0.0001$ both trees) while catechins were typically lowest in the

youngest leaves (age class: $p<0.001$ for both trees). Flavonol glycosides were significantly higher in the younger leaves, although only slight differences between the age classes occurred.

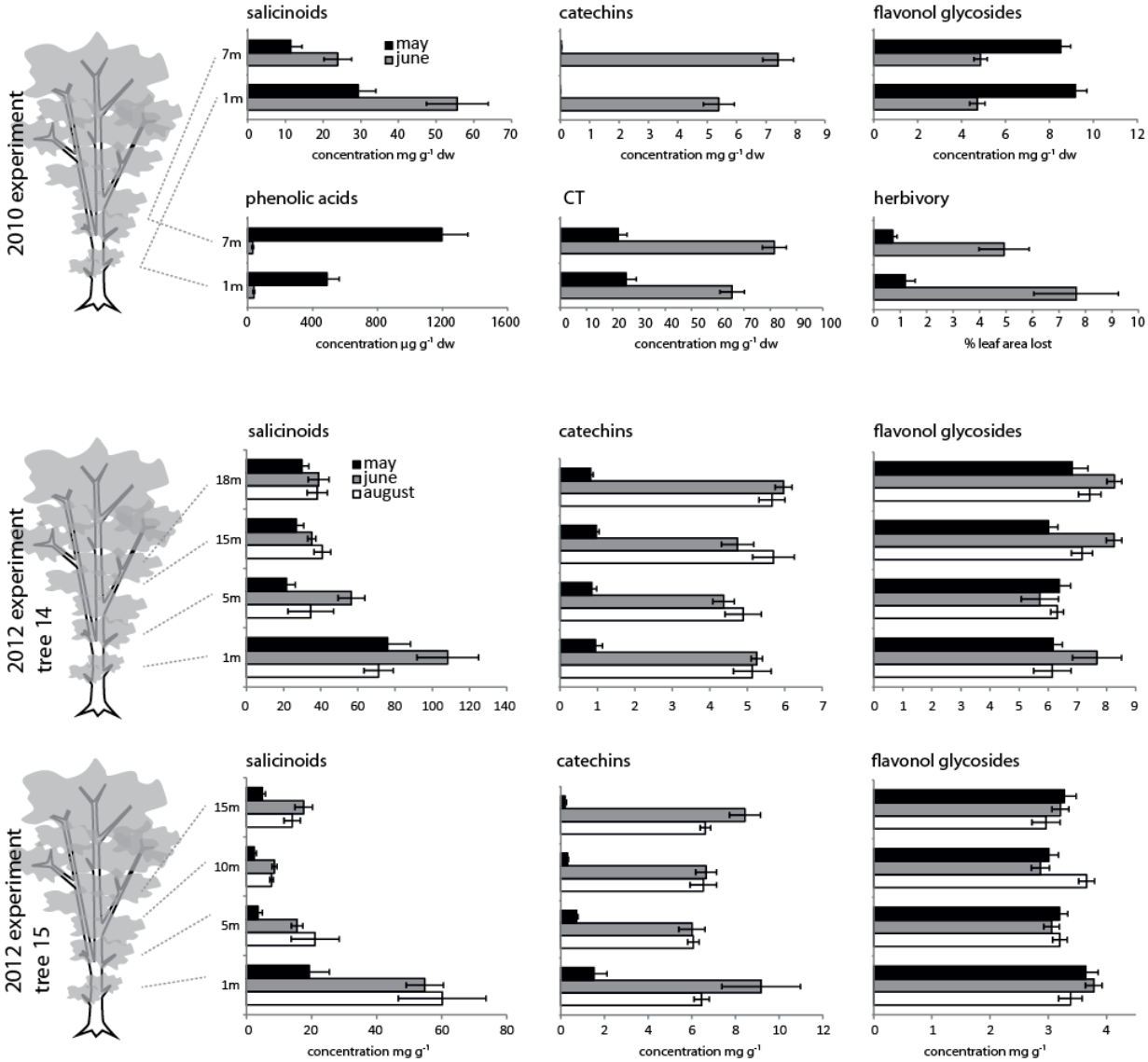

Fig. 2. Height related patterns of leaf parameters in the 2010 (20 trees, 2 heights) and 2012 (2 trees, 4 heights) experiment. Bars in the 2010 experiment (upper panel) represent means ± SE of 20 trees. Means of single trees were calculated from one (herbivory) or three (phenolics) replicates per date, respectively. Each bar in the 2012 experiment represents the mean concentration ± SE of the foliage from 5 apical shoots. The content of every shoot is the average salicinoid content of all leaves of the respective shoot.

Significant effects of date x age class interactions were found for salicinoids and catechins, yet no obvious pattern was evident. Overall the young leaves sustained a higher leaf area loss irrespective if herbivory was measured in relation to the total leaf area (as shown in **Fig. 3**) or as absolute area (in cm^2). For example, in May the young, middle and old leaves of tree 14 exhibited 2.2, 0.6 and 0.7 cm^2 leaf area loss, respectively.

Table 2. Results of linear mixed effect model on height related variation of phenolics in *Populus nigra* trees. Corresponding data are shown in Fig. 2.

2010 experiment

	salicinoids		catechins		flavonol glyc.	
	p	AIC	*p*	AIC	*p*	AIC
height	<0.001	174.5	<0.001	35.9	0.329	1.3
date	0.022	171.2	na	na	0.003	-5.4
height x date	0.286	172.0	na	na	0.145	-5.5

2010 experiment

	phen. acids		CT		herbivory	
	p	AIC	*p*	AIC	*p*	AIC
height	0.128	160.8	0.010	254.7	0.018	250.6
date	0.001	151.0	0.002	247.1	0.005	244.6
height x date	<0.001	122.8	0.017	243.4	0.141	244.4

2012 experiment tree 14

	salicinoids		catechins		flavonol glyc.	
	p	AIC	*p*	AIC		
height	0.069	108.9	<0.001	1.5	0.0616	-28.7
date	<0.001	73.0	0.2505	3.4	0.0043	-35.8
height x date	0.104	73.4	0.4142	9.3	0.0176	-39.2

2012 experiment tree 15

	salicinoids		catechins		flavonol glyc.	
	p	AIC	*p*	AIC		
height	0.007	157.5	<0.001	7.6	0.768	80.5
date	<0.001	88.6	0.227	9.3	0.005	73.8
height x date	0.882	98.3	<0.001	-9.4	0.004	66.9

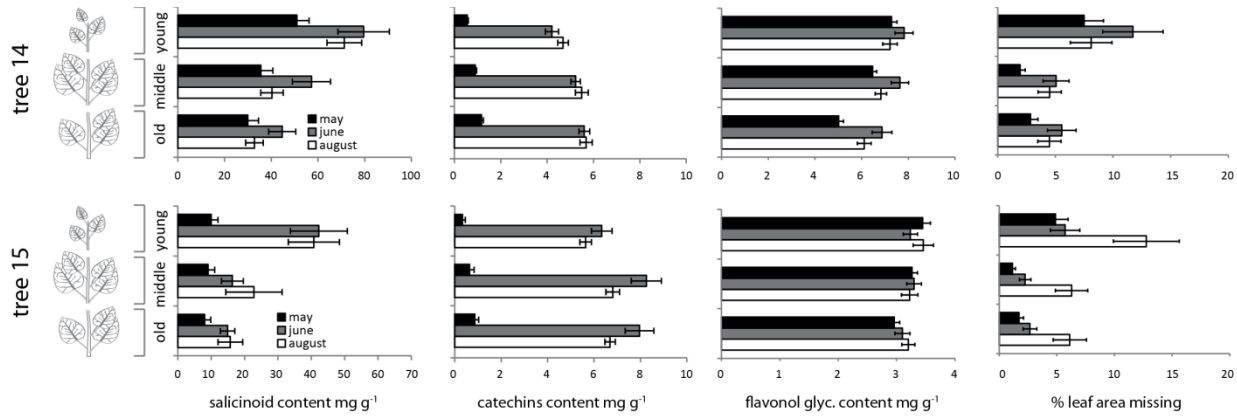

Fig. 3. Ontogeny related leaf parameters measured in two *Populus nigra* trees. Each bar shows the mean content of 20 replicate branches per month ± SE. Leaves across all heights were assigned to three age classes (2 oldest leaves="old", 2 youngest="young", rest="middle").

Table 3. Results of linear mixed effect model on ontogeny-related variation of phenolics in *Populus nigra* trees. Corresponding data are shown in Fig. 3.

		salicinoids		catechins		flavonol glyc.		herbivory	
		p	AIC	*p*	AIC	*p*	AIC	*p*	AIC
tree 14	**date**	0.077	709.6	<0.001	24.7	0.054	71.5	0.284	-122.3
	age class	<0.001	547.0	<0.001	-24.8	<0.001	34.0	0.001	-132.8
	date x age class	<0.001	534.9	0.605	-19.6	0.158	35.4	0.898	-125.8
tree 15	**date**	0.006	451.5	<0.001	144.6	0.815	307.9	0.047	-295.4
	age class	<0.001	375.0	<0.001	86.4	<0.001	295.0	0.011	-300.3
	date x age class	0.032	372.4	0.032	83.9	0.060	293.9	0.861	-293.6

Correlation of phenolics with herbivory and fungal infestation

The elucidation of herbivory or pathogen infestation was considerably complicated by the temporal dynamics of both these stress factors and phenolic content. We decided to analyze the data for simple correlations at the time when the biotic stress was found to be strongest. Thus phenolics were correlated with herbivory in June 2010, after the major leaf area loss was recorded. Levels of all classes of phenolics showed no significant correlation to insect herbivory, as shown for salicinoids in **Fig. 4**. However, in August 2010, when virtually all trees showed signs of massive leaf rust infestation, foliar catechin levels were significantly and negatively correlated with glucosamine (p=0.007).

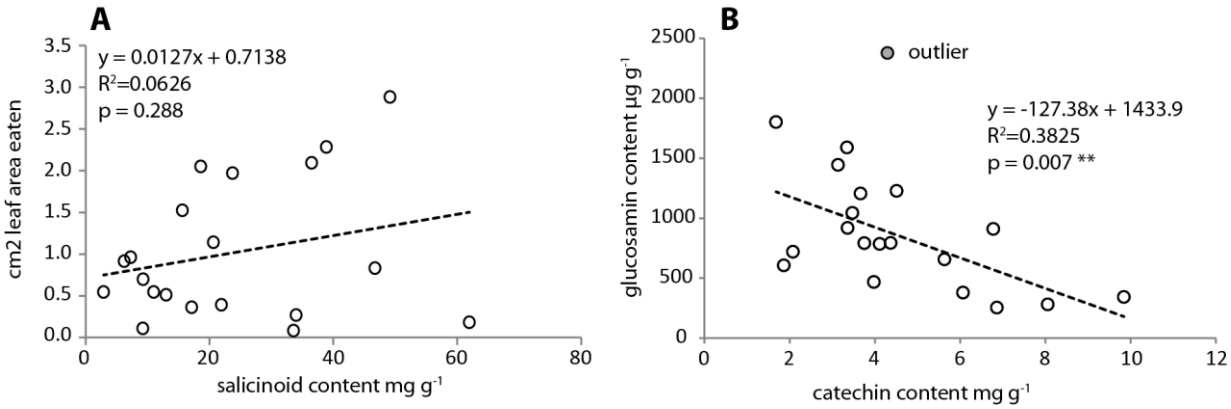

Fig. 4. Levels of herbivory (A) and fungal infestation (B), expressed as glucosamine content, in leaves of *P. nigra* in relation to foliar salicinoid and catechin contents, respectively. Panel A shows cm^2-herbivory correlated to the salicinoid levels as found in June 2010, shortly after the main herbivore activity. Panel B shows the correlation of glucosamine, a component of fungal cell walls, with catechin in August 2010, when fungal infestation was strongest. N=20 trees.

Discussion

Our studies show that overall phenolic levels, herbivory as well as carbon and nutrient levels in black poplar are subjected to substantial seasonal variation. We also document that several phenolics and herbivory differed with respect to the position in the tree crown and along the shoot axis. Chewing herbivore activity (expressed as percent leaf area loss) was highest early in the year, when phenolics were still low, but focused on foliage with the highest levels of salicinoids, which are known to be potent defense compounds. However, an inter-tree comparison of the 20 investigated black poplar individuals indicated no correlation between leaf damage and salicinoid levels, although a marked genotypic variation in the contents of these phenolics was observed. Instead, catechin was negatively correlated with glucosamine, an indirect measure of fungal (*Melampsora* spp.) infestation.

Seasonal patterns

The seasonal abundance of carbon-based secondary metabolites in tree foliage has been studied at various scales in a few perennial species, such as *Populus fremontii*, *Populus angustifolia* and their hybrids (Holeski *et al.* 2012), *Betula pubescens* (Salminen *et al.* 2001; Riipi *et al.* 2004) and *Quercus* sp. (Feeny & Bostock 1968; Covelo & Gallardo 2001; Salminen *et al.* 2004). In all species, the seasonal development of condensed tannins is consistently characterized by a strong increase until June or July, followed by a plateau until leaf fall. This trajectory is in good agreement with our findings in the black poplar population, where condensed tannins increased from 20 mg/g to 80 mg/g DW between May and June. Similarly, the initial levels of salicinoids, the other major class of phenolics in black poplar, were low right after leaf flush and accumulated in the course of summer. This is in contradiction to previous seasonal recordings on foliar salicinoids in a North American poplar species, which tend to document a decline of salicinoids during summer (Wimp *et al.* 2007; Holeski *et al.* 2012). However, our results suggest that black poplar prioritizes growth instead of defense early in the year: in addition to the seasonal trajectory of phenolics, we observed a trade-off between biomass production and salicinoid contents in May (Supplemental 2). Similarly, we found that 70 % of the shoot biomass produced before August was already present in May, indicating a strong early allocation of resources to growth. Trade-offs between growth and salicinoids are well-known from young *Salix myrsinifolia* (Ruuhola & Julkunen-Tiitto 2003), but in mature trees it may be possible that salicinoids are translocated to freshly formed foliage from the bark, which is rich in various phenolics. A survey of the bark of three willow species supports this hypothesis (Förster *et al.* 2010), but our long-term observation of salicinoids in

black poplar bark does not suggest a significant translocation in spring (Supplemental 3). If there is no source for foliar phenolics in poplar, the biosynthesis of salicinoids and condensed tannins may just take too long to allow high levels in leaves immediately.

Interestingly the minor phenolic constituents show seasonal changes different from those of the main compounds of black poplar foliage. The dramatic early season decline of phenolic acids can be related to the role of these compounds in lignification (Ralph 2009) and it seems likely that they are consumed to promote leaf toughness. The immediate presence of flavonol glycosides might be due to their essential function in UV light screening (Agati *et al.* 2013) or a putative role in the regulation of redox processes (Hernandez *et al.* 2009). Catechins are intermediates in condensed tannin biosynthesis (Mellway & Constabel 2009) but follow a specific seasonal pattern, suggesting an additional function other than being merely biosynthetic intermediates.

The seasonal trajectory of the macro nutrients like nitrogen is well conserved across woody plants. Investigations of birch (Haukioja, Ossipov & Lempa 2002; Ruuhola *et al.* 2003), oak (Feeny & Bostock 1968) and trembling aspen (Osier, Hwang & Lindroth 2000) showed that nitrogen or protein levels, respectively, decrease with leaf maturation and the same patterns hold true for black poplar. As nitrogen is an important nutrient, its rapid decline has been suggested to be one reason why many insect species emerge with leaf flush (Feeny 1970). In agreement with this theory we document the major leaf area loss in black poplar during the first 6 weeks after leaf flush. Additionally black poplar appeared to shed severely damaged leaves, as the average leaf area missing tended to decline after June. Despite these complications we argue that the major defoliation of black poplar by leaf chewers occurs early in spring.

Spatial patterns

Secondary metabolite gradients within shoots are frequently observed in poplar and salicinoids typically increase in apical direction, while condensed tannins decrease (Bingaman & Hart 1993; Kleiner *et al.* 2003; Boeckler, Gershenzon & Unsicker 2013). As salicinoids are very potent direct defenses, this pattern has been related to the high value of the apex as site of shoot prolongation and to the higher nutritive value of young leaves, making them more attractive to herbivores. In accordance with this theory we observed that young leaves typically exhibited higher rates of defoliation. Surprisingly the distribution of phenolics along the shoot axis in black poplar was very persistent, although herbivory and leaf formation mainly occurred in spring. Unless this distribution is permanently required, it might be that poplars lack the ability

to recycle large amounts of phenolic end products. Salicinoids at least are turned over rather slowly in intact willow leaves (Ruuhola & Julkunen-Tiitto 2000).

The distribution of secondary metabolites within the tree crown has been rarely studied (but see (but see Karolewski, Jagodzinski & Grzebyta 2011). In two experiments we found that salicinoids were significantly higher concentrated in foliage near the ground vs. higher in the canopy, while the patterns of the other phenolics were less consistent. Concomitant to the elevated salicinoid levels a higher defoliation rate near the ground was recorded. In a flood-plain forest, where trees are usually scattered over large areas, the lower crown part may suffer from higher herbivore pressure due to the dispersion of herbivores from the neighboring vegetation. Additionally, it is conceivable that herbivores overwintering below ground as pupae consistently prefer the closest foliage for oviposition, and do not reach the higher crown. Both factors can be understood as permanent environmental stress in the lower crown and therefore the elevated salicinoid levels may be a pattern of phenotypic plasticity.

Sex dependent patterns

According to our results the abundance of some phenolics and carbon differ between male and female trees in black poplar foliage and bark. Sexual dimorphism in dioecious plants can have detectable impact on the secondary chemistry (Palo 1984), arthropod community (Cornelissen & Stiling 2005; Petry *et al.* 2013) and other traits (Ahman 1997; Dudley 2006) and may originate from the allocation of resources to reproduction in female individuals. In the family of the Salicaceae Nichols-Orians, Fritz and Clausen (1993) found no impact of sex on the phenolics in *Salix sericea* but volatile organic compounds released by flowers of *Salix fragilis*, *Salix myrsinifolia* and *Salix tiandra* (Fussel *et al.* 2007) differed between male and female individuals. It is conceivable that the sex-based differences in the phenolics of black poplar influence the plants or their environment. However, the present knowledge and methodology does not yet allow robust theories and their validation, respectively.

Impact of phenolics on herbivores and fungal infestation

As we know from the literature salicinoids are supposed to be the most potent defense compounds against generalist herbivores in poplar (Lindroth & St Clair 2013; Boeckler *et al.* 2014). Despite an enormous genotypic variation of salicinoids within the investigated trees, we found no early year correlation between salicinoids and leaf area loss. This observation is similar to other field studies, where a protective function of defense metabolites is often not observed (Moyes 2000). Typically this pattern is explained by the presence of specialist

herbivores (Bidart-Bouzat & Kliebenstein 2008; Macel & Klinkhamer 2010), which are not harmed or even stimulated by direct defense compounds. Alternatively, the low early year salicinoid levels observed in our study may have been just too low to provide significant protection, or the high levels of nutrients compensated for possible adverse effects.

In contrast, the pathogenic leaf rust (*Melampsora* sp) was negatively correlated with catechin. Anti-fungal activity of condensed tannins has been suggested earlier based on the observed resistance of CT over-expressing poplar against *P. tremula x tremuloides*. However, the transgenic trees were also higher in catechin, a putative precursor in CT biosynthesis (Boeckler *et al.* 2014). Fungicidal effects of phenolics were also reported from spruce, where the pathogen *Ceratocystis polonica* is negatively affected by astringin (Hammerbacher *et al.* 2013). However, the role of secondary metabolites against pathogens is not well-established and the underlying mechanisms are unknown. Therefore further experiments are required to better evaluate the efficacy of phenolics against fungi and to improve our understanding of the mode of action. Black poplar is very susceptible to leaf rust and we observed that most trees at our field site were massively infested for five successive years. Although not directly lethal, this pathogen certainly weakens the trees by causing premature leaf fall and high catechin genotypes may have a potentially higher resistance.

In summary, our study shows that phenolics in black poplar show compound class specific spatiotemporal variation. Improved methods are needed to disentangle the complex relationship between the folivores or pathogens of black poplar and the chemistry of their host plants.

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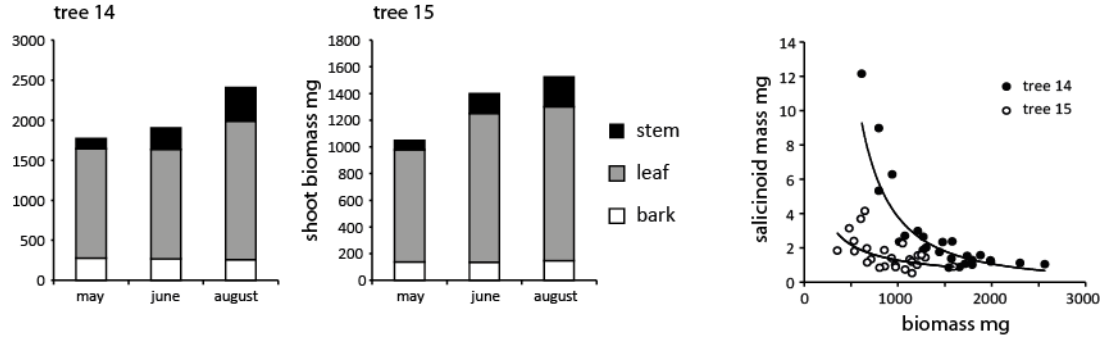
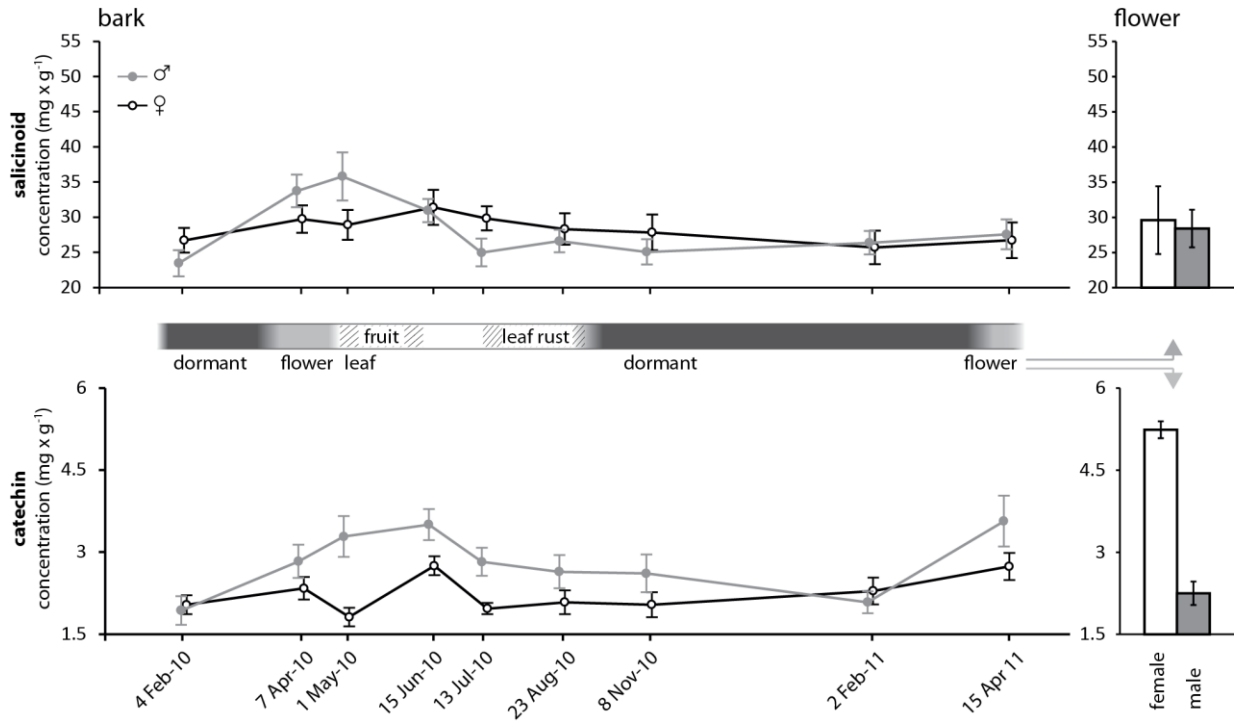
Appendix

Supplemental 1: Analysis of glucosamine 1 ml of 8 N HCl was added to 10 mg of sample and the mixture was boiled for 90 mins at 99 °C. The cold sample was centrifuged and 10 µL of the supernatant was transferred into a 1.5 ml vial and thoroughly mixed with 10 µL 8 N NaOH,180 µL borate buffer at pH=7 containing 2.5 µg/ml mannosamine and 200 µL of an 30 mM FMOC solution in ACN. After 5 min 800 µL n-heptane was added and the two phases were thoroughly homogenized. After phase separation a 150 µL aliquot of the aqueous (bottom) phase was used for LC-analysis and separated on Agilent 1200 series chromatographic system equipped with a Agilent XDB-C-18 column (4.6 x 50 mm, 1.8 µm). Gradient elution with 0.05 % aqueous formic acid (A) and acetonitrile (B) was applied using the following parameters: 10% B (0-0.5 min), 10-66 % B (0.5-9.0 min), 100 % B (9.0-10.0 min), 10 % B (10.0-13.0 min). Eluted compounds were detected on an API 5000 LC/MS/MS mass spectrometer (Applied Biosystems, Carlsbad, CA, USA) operated in negative ionization mode and using multiple reaction monitoring (MRM). MRM-parameters: (parent ion *m/z* → product ion *m/z*; declustering potential [V], collision energy [V]): mannosamine FMOC adduct (IS) and glucosamine FMOC adduct (402.1→178.1; -96, -81). Quantification was accomplished using the internal standard method.

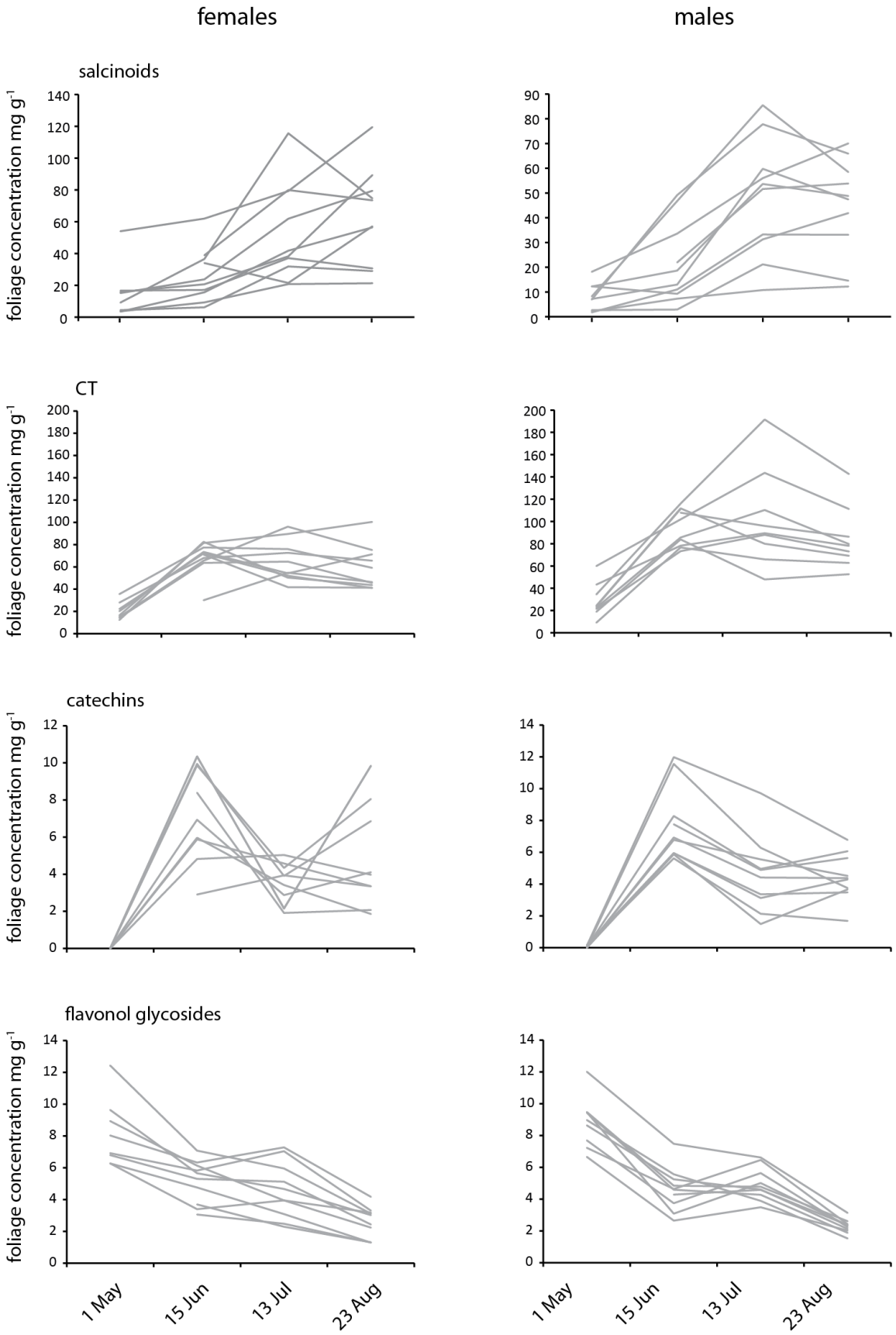
Supplemental 2: Biomass gain of shoots harvested in the 2012 experiment. Regression of biomass against the total amount of salicinoids per shoot measured in May 2012.



Supplemental 3: Seasonal trajectories of salicinoid and catechin concentrations in *Populus nigra* bark and flowers. Each data point in the bark graph represents the mean ± SE of 10 individual trees that were sampled in triplicate. The bar in the middle indicates the approximate vegetative state and occurrence of Melampsora infestation. Panels on the right hand side show the concentrations in flowers harvested on April 15th 2011.



Supplemental 4: Seasonal development of foliage phenolics in single trees. Each tree is represented by the average of three replicates per time point.



4. Discussion

4.1. Condensed tannins have no broad-spectrum bioactivity against herbivores

Salicinoids and condensed tannins are the most abundant phenolics in *Populus tremuloides* (Donaldson *et al.* 2006) and this pattern also applies to the hybrid *P. tremula* x *tremuloides* trees used in our experiments (Manuscript I). Both compound classes occur in equally high concentrations and have been proposed to function as direct defenses against herbivores. While there is little doubt that salicinoids have a broad spectrum anti-herbivore activity against generalists, it is unclear if condensed tannins exhibit such properties. In the literature, negative correlations between condensed tannin content and herbivore performance (Mutikainen *et al.* 2000) or abundance (Forkner, Marquis & Lill 2004) stand in contrast to other studies that question any influence on insect herbivores (Hemming & Lindroth 1995; Ayres *et al.* 1997). Our experiments document that gypsy moth caterpillars experienced greater weight gain and faster development when fed with condensed tannin over-expressing transgenic poplar. However, since these transgenic poplar lines showed altered salicinoid phenotypes accompanying condensed tannin over-expression, it was not possible to separate the contribution of both types of compounds to gypsy moth performance. As matter of fact, gypsy moth caterpillars performed much better on trees that were high in condensed tannins (approx. 16 % DW) and low in salicinoids (approx. 6 % DW) than on trees that were low in condensed tannins (approx. 8 % DW) and high in salicinoids (approx. 9 % DW), and we therefore must conclude that condensed tannins are beneficial or salicinoids are detrimental, or both. Positive correlations of condensed tannins with herbivore performance parameters have been sporadically reported (Rossiter, Schultz & Baldwin 1988) and may be related to their modest reducing properties that can compromise the effect of phenolics that are oxidizing (Barbehenn *et al.* 2006), such as bioactivated salicinoids (see below). Alternatively, condensed tannins levels are often negatively correlated with salicinoid levels, so that any effect on herbivores can be an artefact of covariance (Hemming & Lindroth 1995). In any case, our results do not support the notion that condensed tannins have anti-herbivore activity against the generalist gypsy moth. Considering the proposed modes of phenolic action, oxidation of condensed tannins to quinones appears unlikely, as their bulky structure should impede enzymatic action by polyphenol oxidases. Protein precipitation is also unlikely as the typically alkaline gut pH of lepidopterans is likely to deprotonate phenolic OH groups and thus prevents protein binding by

hydrogen bonds (Appel 1993). Astringency is conceivable in herbivores with a neutral or acidic gut pH, but a general astringent role for condensed tannins as suggested by Feeny (1970) is no longer accepted.

There are some hints in the literature that condensed tannins function in pathogen defense (Miranda *et al.* 2007), and our results in manuscript V indicate such a role for catechin, a monomeric building block of condensed tannins. Flavan-3-ols like catechin or condensed tannins possess an aryl-C_3-aryl skeleton which resembles diaryl-ethenes known to be anti-fungal (Hammerbacher *et al.* 2013). If condensed tannins were primarily pathogen defenses, this could justify the remarkable resource allocation to their production and their inducibility by herbivore damage, which may allow pathogen entry.

4.2 Salicinoids are toxic and can be partially metabolized in the gypsy moth

The performance experiment presented in manuscript I indicated that salicinoids affect gypsy moth food choice, developmental times, weight gain and mortality, but the concurrent changes in condensed tannins did not permit an unambiguous attribution of these effects to either of the two phenolic classes. Irrespective of condensed tannins, the literature leaves little doubt that salicinoids are highly efficient direct defenses in the poplar system (reviewed in manuscript II). However, the *P. tremula* x *tremuloides* hybrids produce three different salicinoids, namely salicin, salicortin and tremulacin, which exhibit variable and structure-dependent bioactivity (Lindroth 1991). Like all salicinoids, salicortin and tremulacin are composed of modules including a salicin core structure, an HCC (1-hydroxy-6-oxo-cyclohexen-2-en-1-carboxylic acid) building block (salicortin and tremulacin) and a benzoyl moiety (only tremulacin). Diet supplementation experiments have shown that the primary toxicity of salicinoids resides in the HCC moiety, while salicin and benzoic acid are harmless in natural concentrations (Lindroth 1988a; Lindroth & Peterson 1988; Lindroth, Scriber & Hsia 1988). Despite these divergent effects of salicinoids on caterpillar performance parameters, there is little knowledge about the metabolism, bioactivation and detoxification in the digestive system. These processes were our main concern in manuscript III, where we described five previously unknown detoxification products. Our experiments documented that salicinoids are almost entirely degraded to salicin and the corresponding organic acids during passage through the gypsy moth gut, which is in agreement with previous reports from *O. brumata* (Ruuhola, Tikkanen & Tahvanainen 2001). Salicin and benzoic acid apparently do not experience further conversion as they were recovered unaltered or as conjugates, respectively. In contrast, HCC is very likely converted to catechol, as indicated by the presence of two catechol containing conjugates in the feces. This

conversion was formerly suggested (Clausen *et al.* 1989) and supported by feeding studies in insects (Ruuhola, Tikkanen & Tahvanainen 2001) and human endothelial cells (Knuth *et al.* 2011). Our subsequent performance assays using diet supplemented with the degradation products showed that catechol has adverse effects on the gypsy moth just like salicin when fed in concentrations that reflect those of its salicinoid precursors. Benzoic acid, however, had no detectable effect.

In summary, my results raise to two major questions, which will be shortly addressed in the following two paragraphs: First, it is important to follow up the fate of the salicinoid building blocks that were not recovered as conjugates in the feces. It would be particularly interesting to learn if salicin and catechol undergo further bioactivation and if the end products of such reactions ultimately lead to the physiological effects observed in the bioassays. Second, it would also be worthwhile to investigate the actual location of the conjugation reaction in the insect, the enzymes involved in conjugation, and its metabolic cost. Moreover, two of the salicinoid metabolites described, namely salicin phosphate and catechol glycoside phosphate, deserve more attention as they have not been previously described and may be part of a novel detoxification cascade.

The mode of action of salicin and catechol

The glycosidic nature of salicinoids suggests that these compounds undergo a similar bioactivation as the other glycosidic defense compounds mentioned in **1.2**. However, our results have shown that the degradation is likely initiated by a spontaneous ester hydrolysis in the gut of Lepidoptera (Ruuhola, Julkunen-Tiitto & Vainiotalo 2003), where alkaline conditions are the rule (Johnson & Felton 1996). Based on our bioassays, we assume that the salicin and catechol released are the major bioactive metabolites. How can these two compounds harm the gypsy moth and lead to the physiological effects observed earlier? In our current state of knowledge we can only speculate whether both compounds remain in the digestive system or reach other organs. Irrespective of its location, salicin could be bioactivated by enzyme-catalyzed deglucosylation. However, the liberated saligenin has not yet been subjected to toxicity tests with insects. It is also not known if the monophenolic saligenin can be further bioactivated, for example by hydroxylation and subsequent oxidation by polyphenol oxidases. Data from vertebrate herbivores suggest that no such reaction occurs since saligenin is readily conjugated with glucuronic acid and excreted (McLean *et al.* 2001). Since insects rely on glycosylation instead of glucuronation (Ahn, Vogel & Heckel 2012) some of the salicin found in the gypsy moth feces may have been transiently cleaved and re-conjugated with

glucose. Overall, there is some evidence that digestive glucosidase activities are decreased upon salicinoid exposure (Hemming & Lindroth 2000) suggesting that salicin deglycosylation is unfavorable. In theory, this down-regulation of digestive enzymes could be an avoidance strategy to reduce the formation of toxic metabolites despite hampering the acquisition of sugars from the diet (see **1.4**). However, the further reactions of salicin, its biological targets and their location require further evaluation.

In contrast to salicin, catechol is a di-phenolic and can be immediately oxidized to *o*-quinone in a spontaneous reaction (Pearl & Darling 1971) or via catalysis of endogenous plant polyphenol oxidases (i.e. laccases and catechol oxidases) and peroxidases (Haruta, Pedersen & Constabel 2001; Pourcel *et al.* 2007). However, both the enzymatic and the spontaneous reaction require oxygen and so could be impeded by the anoxic conditions in caterpillar midguts (Constabel & Barbehenn 2008). The detection of *o*-quinone is difficult since this species is very electrophilic and short-lived. Therefore it is currently unclear if the oxidation of catechol actually occurs. It is theoretically possible to use electron paramagnetic resonance spectroscopy to detect semi-quinone radicals, which are formed as intermediates when catechol oxidation is carried out by laccases or peroxidases, (Barbehenn, Poopat & Spencer 2003). However, catechol oxidases can convert catechol to *o*-quinone without intermediate radical formation (Pourcel *et al.* 2007) and therefore electron magnetic resonance spectroscopy cannot ultimately disprove catechol oxidation. The easiest way to confirm *o*-quinone formation may be the detection of adducts resulting from the reaction with biomolecules, which is supposedly the basis of their bioactivity, but quinone-chemistry is complex and involves various types of electrophilic and radical reactions. One possible metabolic target are free amino acids and proteins which can undergo Michael additions, Strecker degradations and imine formation when exposed to quinones (Bittner 2006), reactions that significantly deteriorate the nutritional quality and are important in food browning (Friedman 1996). In fact, I observed that the feces of gypsy moth fed artificial diet supplemented with catechol (manuscript III) were darker than feces from control diets without catechol and therefore such reactions may have occurred in the digestive system without PPO action. Reaction of salicinoid products with amino acids is supported by the observation that gypsy moth caterpillars can tolerate higher amounts of salicinoids when their diet is enriched with casein (Hemming & Lindroth 2000) which could compensate for the putative loss of dietary amino acids caused by the reaction with quinones. Apart from dietary constituents, endogenous caterpillar molecules may also be the target of the quinones. The digestive enzymes of this herbivore could be attacked and inactivated by quinone addition. Alternatively, quinones could react with the double bonds of unsaturated fatty acids in a Diels-

Alder reaction manner and thus affect the integrity of membranes, which may be an explanation for the lesions observed in southern armyworm midgut membranes upon exposure to dietary salicinoids (Lindroth & Peterson 1988). In summary, there is much evidence that catechol reacts to form *o*-quinones in insect herbivores, but unless researchers succeed in the detection of a specific adduct with a biomolecule, the molecular basis of salicinoid toxicology will remain hypothetical.

Detoxification activities in the gypsy moth

Previous observations of insect enzyme activity changes in response to salicinoid-containing diet indicated that esterases and glutathione transferases are involved in the detoxification of these phenolics (Hemming & Lindroth 2000). However, my results disagree with this theory and suggest instead that a completely different suite of enzymes is responsible. The data in manuscript III indicate that salicinoid metabolism releases benzoic acid, salicin (possibly saligenin) and catechol, which are then converted to conjugates. With respect to benzoic acid two reactions are presumably necessary for the conversion to hippuric acid. In humans, benzoic acid is metabolized in the mitochondria and activated with CoA before being transferred to a glycine molecule via catalysis of glycine N-acyltransferase (Knights, Sykes & Miners 2007). Hippuric acid formation has been observed in different organisms and may be a conserved strategy of invertebrate and vertebrate herbivores to metabolize benzoic acid. In contrast, the catechol formed upon salicortin ingestion is typically sulfated in rats and humans (Knuth *et al.* 2013), while our findings indicate that lepidopteran herbivores prefer glycosylation likely catalyzed by UDP glycosyl transferases. These enzymes are suggested to be widespread detoxification enzymes in insects (Ahn, Vogel & Heckel 2012), although only very few conjugations of direct defense compounds are known so far (Després, David & Gallet 2007). Interestingly glycosylation is also a major reaction of phenolics in plants (Bowles *et al.* 2006) and catechol glycoside is known from poplar (Morse *et al.* 2007). The conversion rates measured in manuscript III indicate that catechol glycosylation is rather inefficient in the gypsy moth, if we assume that all of the ingested HCC is transiently present as catechol. On the other hand, the pool of free catechol in the feces appeared small, although a sensitive quantification was not feasible with the LC/MS method applied. The low efficiency of catechol glycosylation may be a consequence of having various metabolic fates, including oxidation to *o*-quinones. Overall, much more research is needed to understand the metabolism of salicinoids and other plant defenses.

The detection of salicin phosphate and catechol glycoside phosphate reported in manuscript III is a novel aspect of our work. Phosphorylations are typically carried out by kinases, but the location and purpose of these reactions in the gypsy moth are not clear. Preliminary results indicate that the phosphorylated metabolites are present in the hemolymph and we therefore speculate that the phosphate group could serve to facilitate transport back into the gut lumen. Further experiments are on the way to elucidate the role of phosphorylation and to clarify if the detoxification products observed in the gypsy moth occur in other arthropod herbivores. In addition it would be interesting to know if these conjugations also occur for other direct defense compounds or xenobiotics in general. If these conjugations are widespread in insects, gene expression studies with salicinoid exposed gypsy moths could facilitate the identification of enzymes involved.

4.3 *Populus nigra* produces a rare salicinoid and various other phenolics

The salicinoid metabolism experiments just described were carried out in the laboratory on mixtures of standard compounds. However, such experiments are only partly representative of salicinoid diversity in poplar. Thus we investigated a native black poplar (*P. nigra*) population consisting of mature trees growing in a natural flood plain and associated with a complex herbivore community (Boeckler, personal observation). Work on *P. nigra* and various species of *Populus* is important since the overwhelming majority of studies exploring the role of phenolics in the herbivore defense of poplars were conducted almost exclusively with one species native to North America, namely *Populus tremuloides* (summarized in Lindroth 1991; Lindroth & St Clair 2013). In addition, work on native trees is necessary since previous studies were often conducted in greenhouse or common garden scenarios with young trees typically grown from stem cuttings and only a handful of model herbivore species. The primary concerns in our field experiments were to explore the spatiotemporal heterogeneity of phenolic abundance and possible feedbacks on herbivory. Initially, we determined the phenolic chemistry of black poplar, which was not previously investigated with modern techniques (but see Thieme & Benecke 1971).

Phytochemical analysis showed that black poplar foliage contains a complex mixture of phenolics. We focused on the major constituents and verified their identity with authentic standards or by NMR analysis of compounds purified from plant material (manuscript IV and V). In summary, eleven individual phenolics and condensed tannins were identified and routine quantification protocols established. Together, these compounds include representatives of four

of the five major classes of phenolics in poplar (sensu Mellway *et al.* 2009), namely flavonol glycosides, phenolic acids, salicinoids and flavan-3-ols, but no anthocyanins. A similar set of compounds was found in *Populus tremula* x *tremuloides* by Kosonen *et al.* (2012), but the authors chose another classification of phenolics. A peculiarity of black poplar is the production of the salicinoids nigracin and homaloside D, which are OH-substituted derivatives of tremuloidin and tremulacin found in *P. tremuloides*. Both compounds appear to be specific to black poplar, although homaloside D is also known from *Homalium ceylanicum* (Ekabo *et al.* 1993), a tropical representative of the Salicaceae. Despite these differences, the phenolic chemistry in black poplar has sufficient resemblance to congeneric species, so that the conclusions have wide applicability.

4.4 Phenolics in black poplar are not per se inducible

As presented in manuscript IV, gypsy moth herbivory did not cause a significant increase in phenolic levels. Although this observation is widely corroborated by the literature (Osier & Lindroth 2001), induction of phenolics has been occasionally reported in other studies (Clausen *et al.* 1989) and may occur under certain conditions. Based on our results, one might ask why salicinoids, if they function in herbivore defense, are not generally inducible but rather produced constitutively in high amounts? Poplars make use of induced defenses since following herbivory they emit volatiles attractive to parasitic wasps that are enemies of their herbivores (Danner *et al.* 2011; Clavijo McCormick 2013). However, in contrast to salicinoids, volatiles are effective at much lower doses (Clavijo McCormick, Gershenzon & Unsicker 2014) and concentration increases by several orders of magnitude can be achieved at comparatively little cost of resources. In contrast, salicinoids are already present at high constitutive levels, approximately 5-10 % of dry weight, and additional synthesis would consume a significant amount of resources and might be too slow to harm herbivores that can change their feeding site. A systemic induction in adjacent, undamaged plant parts can increase defensive posture (Shah 2009), but requires even more resources. Thus the resource need may render salicinoid defenses unsuitable for induction. However, when phenolic levels are initially low, as for example in a specific developmental stage, induction could be more cost-effective. The data presented in manuscript IV were obtained from trees sampled in July (2010 study) and August (2009 study), when high amounts of salicinoids had already accumulated based on the seasonal profile (see below). In spring, when the levels are low, induction may more readily occur. Another interpretation of the lack of salicinoid induction is that the identity of the

attacker may be critical in influencing the plant response. Preliminary studies from our lab indicate that herbivores, such as galling aphids and bark beetles, can induce salicinoids in young trees. Perception of the attacker's identity could be mediated by herbivore specific elicitors (Diezel *et al.* 2009) and a recent study by Clavijo McCormick *et al.* (in submission) shows that the feeding mode and the resulting damage patterns also influence the induction of volatiles. These data as well as the diverse outcomes of studies in the literature (e.g. Barbehenn & Constabel 2011) indicate that induction of phenolics *per se* is not impossible despite the absence of this phenomenon in our study of mature *P. nigra* attacked by gypsy moth larvae.

4.5 Phenolics in poplar vary seasonally and spatially and between genotypes

Seasonal variation

Although the phenolic levels in black poplar were not altered by herbivore feeding, they showed characteristic temporal and spatial variation. Seasonal trajectories of phenolics are well-known from tree species other than poplars. The work of Feeny (Feeny & Bostock 1968; Feeny 1970) on phenolics of *Quercus robur* inspired similar experiments using primarily oak (Mauffette & Oechel 1989; Salminen *et al.* 2004), birch (Riipi *et al.* 2004) and other boreal tree species (Ricklefs & Matthew 1982). Investigations before 1990 largely made use of colorimetric tests that detect total phenolics, but, since the establishment of HPLC methods, discrimination among compound classes is now possible. The more recent studies have documented that the overall quantity of condensed tannins increases over the growing season, while other phenolics often decrease (Riipi *et al.* 2004; Salminen *et al.* 2004). In manuscript V we demonstrated that the overall phenolic content of black poplar leaves increases over the course of the vegetation period. This pattern is driven by the salicinoids and condensed tannins, which are highly abundant but start at very low levels in young leaves. This phenomenon, and the negative early year correlation between biomass production and salicinoid content (manuscript V supplemental), suggest that poplar prioritizes growth over defense immediately after bud break. Rapid growth and the establishment of large amounts of secondary metabolites have been proposed to be mutually exclusive due to resource limitations (Herms & Mattson 1992). However, the general validity of this model has been questioned, and several studies have shown that both processes can occur at the same time (Siemens *et al.* 2002; Arnold & Targett 2003; Massad *et al.* 2014). Nevertheless, negative correlations between salicinoid and biomass levels of young black poplar trees are documented in the literature (Ruuhola &

Julkunen-Tiitto 2003; Hale *et al.* 2005), supporting the idea of an early-year trade-off between growth and phenolics. It may be surprising that growth has such a priority even in mature trees, which are less likely to be in competition than younger or smaller plants. Additionally, low levels of salicinoids in spring contradict the widely accepted belief that young tissue is particularly well defended with these compounds (manuscript II). On the other hand, old-growth trees can tolerate moderate levels of herbivory (Lindroth & St Clair 2013) and therefore high concentrations of direct defense in very young leaves may be unnecessary under normal herbivore pressure. In young leaves, it is also conceivable that resources available for phenolic production are dedicated to the production of flavonol glycosides and phenolic acids, which we found to be present at high levels in spring. Flavonols are known to be important UV-screens and antioxidants and such functions might give them a higher priority than salicinoids or condensed tannins. Phenolic acids may be intermediates in lignification (Ralph 2009), and increasing the toughness of young leaves might be more important to their protection against herbivores than secondary metabolites. Instead of *de novo* biosynthesis, a possible source of salicinoids for developing leaves is the bark of poplars, which can contain remarkable amounts of salicinoids that may be translocated to the foliage. However, it is yet unclear if complex salicinoids can be transported. According to our data, the salicinoids levels in the bark are comparatively stable, and therefore translocation does not appear to play a major role in salicinoid accumulation in developing leaves.

Spatial variation

Although the overall phenolic content of shoots was low in spring, we found that some plant parts contained more of these compounds than others and this pattern persisted over the investigated period. Uneven distributions of plant secondary metabolites in different organs are known from other plants and are believed to depend on their risk of herbivory and value to the plant (McKey 1974; Gershenzon & Croteau 1991; Hamilton *et al.* 2001). Plant parts that are particularly attractive to herbivores are typically better defended than other parts that are unlikely to be attacked (=risk), while plant parts that are particularly important for reproductive fitness, such as seeds or the shoot apex, often exhibit higher levels of defenses (=value). The theory applies well to the patterns that we observed in black poplar and may be particularly useful to explain the variation in salicinoids, which are the major defense compounds *vide supra*. For example, the elevated salicinoid content of foliage of the lower vs. upper crown could be due to risk, since this foliage exhibits a higher rate of herbivory. The increasing salicinoid gradient along the shoot axis from the base to the apex is due to both,

increased risk and value, as the apical bud is the site of elongation (high value) and more apical tissues are preferred by herbivores due to their nutritional value (high risk). The salicinoid gradient along the shoot axis has often been related to leaf ontogeny (manuscript II), but most leaves are produced within a relatively short time frame and should be therefore similar in their developmental status. Possibly the shoot salicinoid pattern is set early in the year and persists as poplar lacks the ability to catabolize salicinoids. However, there is recent evidence for a modest turnover of these compounds (Ruuhola & Julkunen-Tiitto 2000; Massad *et al.* 2014). In summary, phenolics in poplar are constitutively present and, except for the seasonal development, comparatively static. Phenolic involved in anti-herbivore defense appear to be found at higher levels in plant organs that are more likely to be attacked.

4.6 Implications of spatial and temporal heterogeneity of salicinoid for herbivores

Our findings suggest that phenolics are predominantly constitutive in poplars and attacking herbivores are likely to encounter a basal concentration of phenolics. These basal levels, however, are subject to spatial and seasonal variation so that the timing and the site of attack significantly influence the phenolic content of the diet. Additionally, the substantial phenolic content variability between black poplar individuals suggests a strong genotypic background.

In an early study with *Quercus robur* it was recognized that the seasonal increase of foliar condensed tannins is accompanied by decreasing levels of nutrients and these factors were held accountable for the higher density of herbivore species in early spring than later in the season (Feeny 1970). However, some studies do not support the idea that leaf chemistry is a major determinant of insect performance (Mauffette & Oechel 1989) or show annual phenolic trajectories deviating from the pattern mentioned above (Salminen *et al.* 2004). Our investigation of 20 representatives of the largest black poplar population in Germany confirms the previously observed patterns in other tree species and suggests a decreasing food quality of poplar leaves with maturation: A quick decline of nitrogen was observed after leaf flush, along with an accumulation of salicinoids (and total phenolics) over the vegetation period. In addition, a rapid decline of phenolic acids, which are used in lignification (Ralph 2009), will result in an increase in leaf toughness. In contrast, the elevated levels of flavonol glycosides in spring may actually improve herbivore diets by serving as dietary antioxidants (Agati *et al.* 2013) that avert the negative effects of prooxidant secondary metabolites. Given these patterns, it is logical that majority of the annual leaf area loss caused by herbivores occurred in young leaves when the food quality is presumably superior. This result is supported by studies

showing that early feeding herbivore species perform significantly better on developing than mature foliage (Ayres & Maclean 1987; Hunter & Lechowicz 1992; Haukioja, Ossipov & Lempa 2002) and even late season species may grow faster when fed young leaves (Schroeder 1986). In oak the rapid developmental changes of physiochemical leaf parameters have been shown to be more important determinants of food quality than the genotypic variation (Ruusila *et al.* 2005).

Despite this coherence of seasonal phenolic trends in many woody species and the persuasive correlation of increased phenolic content with increased defense potential, it has to be recognized that single phenolic compounds are often not directly related to herbivory. For example a comprehensive study investigating the influence of various leaf parameters (including leaf age and phenolics) of birch on the performance of a single herbivore species showed very complex and interactive effects (Haukioja, Ossipov & Lempa 2002). When we compared the herbivory rates of different poplar genotypes, those with high levels of salicinoids sustained equal amounts of herbivory as those with lower levels, although salicinoids are adverse to generalists (see above). Similar observations are known from field studies with other model systems and are typically explained by the presence of specialized herbivores (see introduction) which feed regardless of the presence of certain defense compounds to which the herbivore is adapted. Alternatively, non-lethal doses of secondary metabolites may lead to a reduced food conversion and induce compensatory feeding of generalists (see supplemental manuscript I). The lack of benefit of plant defenses shown in field studies is a principal dilemma for the current concept of plant defense, as it suggests that the production defense metabolites incur only costs and confer no advantage under natural conditions, where herbivore communities are often complex. Thus the classical expectation of an immediate herbivory reduction by direct defenses may too be simplistic to explain the processes occurring in natural ecological networks. New approaches that consider multiple herbivore species and plant benefits other than a mere reduction of leaf area loss may help to improve our understanding of how plant defenses function in nature.

5. Future perspectives

In this dissertation the toxicity of plant salicinoids in a generalist herbivore were demonstrated and the principal detoxification reactions of the herbivore described. In addition, many phenolics of black poplar were identified, and confounding patterns of spatial and temporal abundance were documented. Thus both, the black poplar and the gypsy moth were established as research platforms to study the chemical ecology of phenolics *in planta* and their detoxification in herbivores, respectively. The following paragraphs will shortly address the various opportunities for further research.

On the plant side, it would be interesting to show what causes the spatial salicinoid heterogeneity in the tree crown and whether this has an effect on herbivory. As we showed that short term bouts of herbivory do not induce increases in salicinoid content, typical defense signaling does not seem to be involved and other processes must regulate the levels of salicinoids. If we could find out how this regulation works, we might discover a new aspect to the control of defenses beyond wound signaling and ontogeny. On the other hand, it has to be demonstrated that the variable salicinoid levels actually affect herbivores and benefit the plant. Intra-plant variation may be used to test the performance of herbivores on foliage with an identical genetic background. Especially the difference in salicinoids with plant height may serve to demonstrate the effect of salicinoids if the variation in other secondary metabolites and nutrients is relatively consistent. This could prove the benefit of direct defense, which has been difficult to achieve so far (**1.3**). If herbivores are actually affected by the spatial heterogeneities of phenolics within the crown, this should be reflected in the composition of the insect community. For example, specialists immune to salicinoids may prefer the lower crown, while susceptible generalists may be more successful at greater heights. Looking for such trends could significantly improve our understanding of how direct defense compounds shape the arthropod community.

On the insect side it is imperative to elucidate if other herbivore species make use of the same processing mechanisms like the gypsy moth. Similarly, it would be interesting to know if the observed mechanisms are involved in the detoxification of other direct defense compounds and other xenobiotics. Generalist herbivores like the gypsy moth encounter various direct defenses and are likely to maintain broad spectrum detoxification machineries. These machineries may also be inducible or variable with instar, and the elucidation and localization of the metabolic processes along with a characterization of the enzymes involved could significantly contribute

to our understanding of plant insect-interactions. Insight in the metabolic disarming of defense compounds also offers the opportunity to develop new insecticides, which are themselves non-toxic but inhibit detoxification processes. Such products could selectively harm herbivores and spare other insects that do not eat the plant.

6. Summary

The battle between plants and their herbivores is fought in many different ways. Defense compounds including toxins and feeding deterrents are major weapons in the arsenal of plants. The defenses of trees in temperate and boreal forests are often phenolic compounds and the genus *Populus* is no exception. Poplars primarily deploy two types of phenolics, condensed tannins and salicinoids, along with minor amounts of phenolic acids, flavonols and other compounds. Salicinoids and condensed tannins have traditionally been the focus of much chemical ecological research. Approximately 40 years of research have evaluated the role of these phenolics classes in the interaction with a handful of vertebrate and invertebrate herbivore species. However, the connections between plant phenolic content and herbivore fitness are often weak, and our understanding of the underlying mechanisms is largely hypothetical. Additionally, many studies were conducted in simplified experimental scenarios with one poplar species (*P. tremuloides*), while knowledge about what occurs under natural conditions and in other species is scarce. The chapters of my dissertation extend the understanding of metabolic processing of phenolic defense in herbivores using the generalist gypsy moth as an example (**Fig. 3**). Additionally, I show that the phenolic defense in mature black poplar trees is subject to spatiotemporal variation, but not inducible by herbivory (**Fig. 3**).

In one of the seminal studies of plant-herbivore chemical ecology published over 40 years ago, condensed tannins were attributed broad spectrum defensive properties against herbivores, but this notion has been subjected to increasing criticism. The experiments presented in manuscript I were designed to evaluate the role of condensed tannins in poplar defense using genetically modified trees with higher contents of these phenolics. My results indicate that condensed tannins are harmless to larvae of the generalist gypsy moth. Instead, the salicinoids, whose levels were also affected by the genetic transformation, but in an inverse manner as the condensed tannins, appeared to be highly detrimental to gypsy moth growth and survival. The bioactivity of salicinoids against herbivores is well-known and has often been addressed experimentally, but was not yet systemically reviewed. I therefore summarized the current knowledge of the chemical ecology of salicinoids in manuscript II and discussed evidence concerning the mode of action of these compounds. In order to improve our mechanistic understanding of the mechanism of salicinoid toxicity, I screened the feces of gypsy moth larvae previously fed three different salicinoids (manuscript III). Five conjugates of salicinoid degradation products were identified and could be unambiguously assigned to specific moieties

of the compounds ingested. Thus my data represents the first *in vivo* observation of salicinoid conjugates in generalist herbivores and provides empirical evidence for the degradation mechanisms predicted earlier. I also describe for the first time two species of phosphorylated sugar conjugates, whose role in detoxification is still not clear. These conjugates were found in lower concentrations when fed to caterpillars previously exposed to phenolics, and thus may be important in the adaptation to dietary phenolics.

In the second part of my thesis I examined various factors that might influence the pattern of phenolics in mature black poplar trees in nature. Screenings of the phenolics present revealed representatives of five different compound classes (salicinoids, condensed tannins, flavonol glycosides, flavan-3-ols and phenolic acids) including four salicinoids. Under the influence of gypsy moth herbivory, very few of these compounds were affected (manuscript IV). However, over the course of the vegetation period, the concentrations of all foliar phenolic compounds varied significantly (manuscript V). Additionally, a remarkable spatial variation was found within the tree crown and within shoots. The temporal development of salicinoid content followed the growth differentiation hypothesis being very low in young leaves during the time of maximum growth, and increasing substantially thereafter. In contrast, the spatial patterns can be better explained by the optimal defense theory with salicinoid levels being particularly high in foliage with a high risk of herbivory.

Given the biological importance of poplar in natural ecosystems and man-made forest plantations, and their use as a model for woody plant species, further knowledge of poplar anti-herbivore defenses is an important goal. My thesis laid the methodological and empirical groundwork for future investigations on the abundant phenolic defenses of poplar, covering their effects on herbivores, the seasonal development and environmental effects on phenolic profiles and the way these defenses are processed by herbivores.

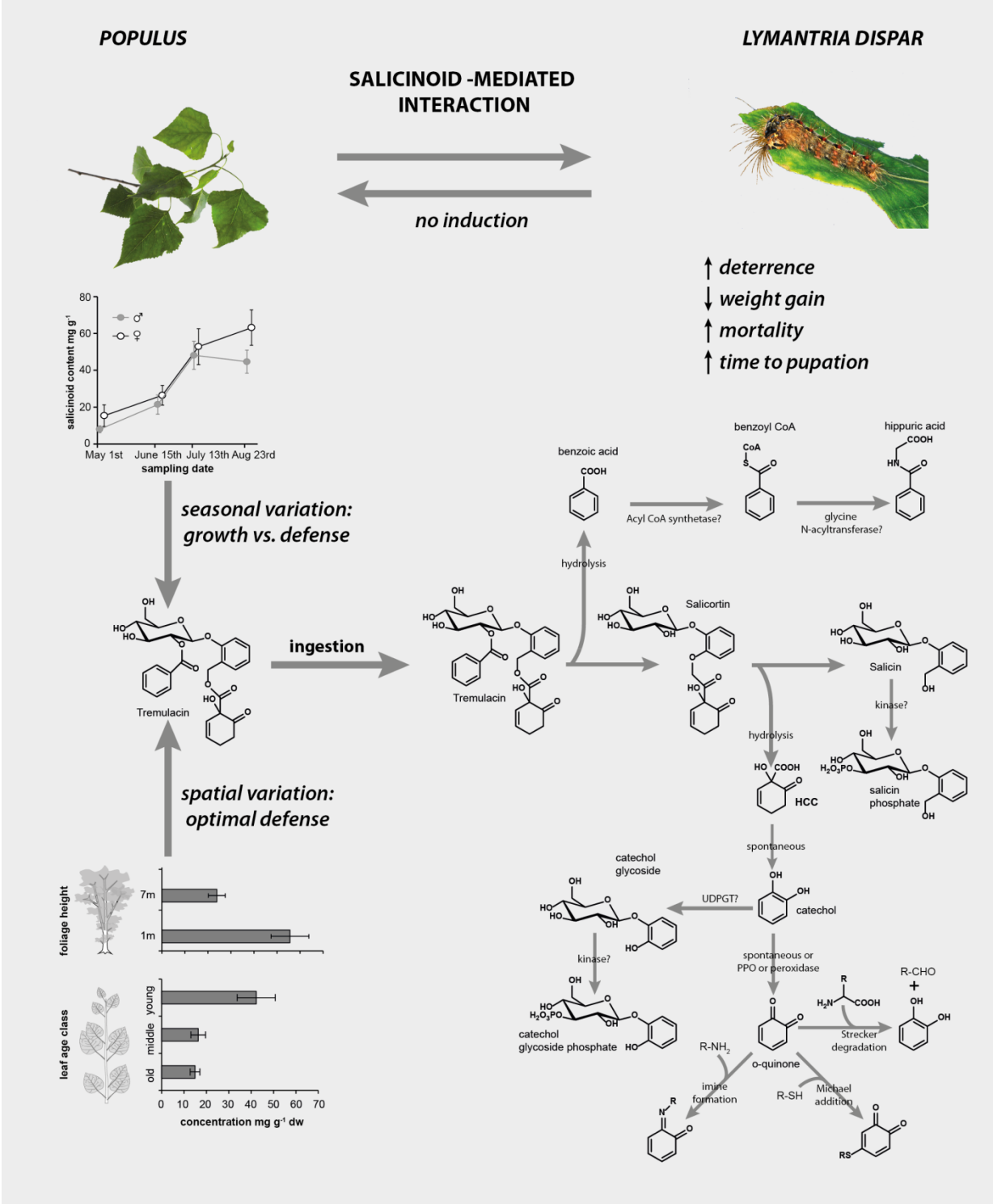


Fig. 3. Depictured key findings of the thesis. Foliar salicinoid levels in poplar are influenced by season and by the position in tree crown or along the shoot axis (left hand side). The ester bonds of ingested salicinoids degrade and the released moieties are conjugated or bioactivated (right hand side).

7. Zusammenfassung

In der Interaktion von Pflanzen und Herbivoren sind pflanzliche Sekundärmetaboliten mit toxischer oder abschreckender Wirkung von zentraler Bedeutung (direkte Verteidigung). In den Holzgewächsen der gemäßigten Breiten, wie beispielsweise Pappeln, fällt diese Funktion häufig von phenolischen Stoffen zu. Pappeln produzieren neben kleineren Mengen von Phenolsäuren, Flavonolen und anderen Verbindungen hauptsächlich zwei Klassen von Phenolen: Salicinoide und kondensierte Tannine. Diese stehen seit etwa 40 Jahren im Interesse der chemisch-ökologischen Forschung, wobei den Salicinoiden auf der Basis von Fallstudien mit Modellherbivoren eine größere Wirksamkeit in der direkten Verteidigung beigemessen wird. Bisher beschränkt sich unsere Kenntnis jedoch auf einfache Zusammenhänge zwischen dem Gehalt spezifischer Verbindungen und Fitnessparametern bzw. Verhaltensweisen von Herbivoren, während die zugrundeliegenden Mechanismen weitgehend unbekannt oder hypothetisch sind. Zusätzlich wurden viele der grundlegenden Studien unter kontrollierten Bedingungen mit einer Nord-amerikanischen Pappelart (*Populus tremuloides*) und einigen assoziierten Insektenspezies durchgeführt, sodass kaum Informationen über die Allgemeingültigkeit der beobachteten Muster in der Natur und in anderen Pappelarten vorliegen. In meiner Dissertation beschreibe ich die Toxikologie von wichtigen phenolischen Verteidigungsmetaboliten, den Salicinoiden, am Beispiel des Schwammspinners. Des Weiteren zeige ich, dass der Gehalt verschiedener Phenolklassen in der Schwarzpappelblättern unter natürlichen Bedingungen deutliche Schwankungen aufweist, die mit der saisonalen Entwicklung und der Lokalisation innerhalb des Baumes zusammenhängen. Allerdings konnte keine Induktion von Phenolen durch Schwammspinnerfraß beobachtet werden.

Als erste phenolische Verbindungen überhaupt wurde den kondensierten Tanninen eine Funktion in der direkten Verteidigung zugesprochen, obwohl neuere Studien diese Eigenschaft in Frage stellen. Die im Zuge von Manuskript I durchgeführten Experimente sollten mit Hilfe von transgenen Pappeln, die höheren Konzentrationen von kondensierten Tanninen aufwiesen, die Rolle dieser Phenolklasse in der Verteidigung gegen herbivore Insekten aufklären. Die Ergebnisse legen nahe, dass kondensierte Tannine keinerlei negative Auswirkungen auf die Raupen des als Modellherbivoren verwendeten Schwammspinners (*Lymantria dispar*) haben. Stattdessen lassen die Ergebnisse eine wesentliche Rolle der Salicinoide vermuten, da diese Phenole als Nebeneffekt der genetischen Transformation ebenfalls veränderte Gehalte aufwiesen und negativ mit Raupenfitnessparametern zusammenhingen. Die biologische Wirksamkeit der Salicinoide und deren Funktion Pflanzen-Insekten-Interaktionen war bisher Gegenstand diverser wissenschaftlicher Studien. In Manuskript II fasse ich die Erkenntnisse

der letzten vier Jahrzehnte mit dem Fazit zusammen, dass das Verständnis der chemischen Ökologie der Salicinoide u.a. durch mangelnde Kenntnis der toxikologischen Prozesse limitiert wird. Diese Prozesse werden in Manuskript III thematisiert, in dem fünf verschiedene Konjugate beschrieben und konkreten Salicinoid-Abbauprodukten zugewiesen werden. Die bis dato erste Beschreibung von Salicinoidkonjugaten in einer herbivoren Insektenspezies liefert die empirische Evidenz für die von anderen Forschern vorgeschlagene Biotransformation dieser Phenole. Weiterhin enthalten zwei der Konjugate eine bisher unbekannte Form von phosphorylierten Zuckern. Die Rolle dieser Konjugate ist derzeit unklar, möglicherweise spielen sie aber eine Rolle in der Nahrungsadaptation, da die fäkale Konzentration wesentlich durch vorherige Phenolexposition beeinflusst wurde.

Im zweiten Abschnitt meiner Dissertation befasse ich mich mit den natürlichen Einflussfaktoren auf den Phenolgehalt von Schwarzpappelblättern. Beim Screening der Phenole dieser Pappelart wurden Verbindungen aus fünf Phenolklassen identifiziert, wobei vier Salicinoide gefunden wurden. Nur wenige dieser Stoffe waren durch Raupenherbivorie geringfügig induzierbar, sodass die Phenole allgemein als konstitutive Verteidigung aufgefasst werden können. Allerdings konnte bei der Untersuchung der Phenole im Jahresverlauf wesentliche saisonale Einflüsse nachgewiesen und zusätzliche eine ungleiche räumliche Verteilung innerhalb des Baumes beobachtet werden. Der saisonale Verlauf der Salicinoide folgt dabei der „growth differentiation balance hypothesis“, da die anfänglich niedrige Konzentration später stark zunimmt. Die räumliche Salicinoidverteilung lässt sich besser mit der „optimal defense theory“ erklären, da eine erhöhte Konzentration in Blattgeweben mit hohem Risiko durch Herbivorenbefall auftrat.

Zusammenfassend beschreibe ich in meiner Dissertation methodische Grundlagen für die Beobachtung der Entgiftung von phenolischen Xenobiotika im Schwammspinner sowie für die Untersuchung der phenol-vermittelten Interaktion von Schwarzpappeln mit deren Umgebung. Mit Hilfe dieser und weiterer Methoden wurde die Relevanz der Salicinoide in der direkten Verteidigung der Pappel sowie deren metabolische Umwandlung in mutmaßlich ungiftige Konjugate erfasst. Zudem umreiße ich die natürlichen Schwankungen des Phenolgehaltes von Schwarzpappelblättern, die für herbivore Insekten von wesentlicher Bedeutung sein könnten.

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