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Salivary cues: simulated roe deer browsing induces systemic changes in phytohormones and defence chemistry in wild-grown maple and beech saplings

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Summary

1. Tree saplings in temperate forests world-wide experience intense browsing from deer and other mammalian herbivores. However, when compared to insect herbivory, our knowledge about the cues that trigger mammalian-induced defence responses in trees is limited.

2. We studied responses of field-grown saplings of European beech (*Fagus sylvatica* L.) and Sycamore maple (*Acer pseudoplatanus* L.) to simulated browsing by (i) clipping apical buds or leaves and (ii) additionally applying roe deer (*Capreolus capreolus* L.) saliva to the cut surface. We analysed induced changes in phytohormones and phenolics in the saplings' remaining buds or leaves, respectively.

3. In both species, jasmonates were activated after clipping of buds and leaves. Importantly, additional saliva application activated salicylic acid in beech leaves and led to increases in cytokinins in beech buds. Saliva application also led to an increased biosynthesis of several hydrolysable tannins (mainly ellagitannins) and flavonols in maple leaves. Condensed tannins, the most abundant phenolics in beech buds and leaves, did not change after either clipping or saliva application. However, clipping with additional saliva application decreased levels of phenolic acids (cinnamic acid derivatives) in beech buds.

4. We conclude that the two tree species perceive and respond to unknown elicitors in the deer saliva, resulting in changes in phytohormone levels and defence-associated secondary metabolites.

5. We suggest that variation in induced defence responses between tree species as well as between buds and leaves is related to differences in morphological traits, which interrelate with chemical traits and result in species-specific strategies to respond to mammalian herbivory.

Key-words: *Acer pseudoplatanus* L. (Sycamore maple), *Capreolus capreolus* L. (European roe deer), *Fagus sylvatica* L. (European beech), induced defence, mammalian herbivory, phytohormones, secondary metabolites, temperate forest

Introduction

Mammalian herbivory can severely impact tree vitality. In temperate broadleaved forests, deer browsing is the main factor that hampers sapling growth and development (Kuijper *et al.* 2010), and can even lead to changes in species composition and ecosystem processes (Horsley, Stout & DeCalesta 2003). Deer browsing markedly impairs sapling performance and mortality mainly through removal of leaves and buds (Augustine & McNaughton 1998; Côté *et al.* 2004; Ammer *et al.* 2010). As opposed to many shrub species, which are equipped with thorns or spines, most temperate deciduous tree species lack such mechanical defence against browsing animals. Whether these trees

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have developed induced defence responses after perception of mammalian herbivores is largely unknown.

Many plants have evolved mechanisms to perceive, and respond to, herbivory (Coley 1983; Bryant *et al.* 1989; Rautio *et al.* 2012). From insect–herb interactions, we know that the first immediate response to herbivory involves the activation of phytohormones, such as jasmonates and cytokinins (Erb, Meldau & Howe 2012; Giron *et al.* 2013). Jasmonates trigger the biosynthesis of defence metabolites in the host plant (Fonseca *et al.* 2009; De Geyter *et al.* 2012). Cytokinins regulate growth responses but also plant interactions with the environment (Sakakibara 2006; Argueso, Ferreira & Kieber 2009), inducing the production of defence metabolites (Großkinsky *et al.* 2011). Although trees' responses might be more complex due to their modular growth as well as ontogenetic and seasonal effects (Laitinen *et al.* 2005; Boege *et al.* 2007), the first chemical responses following foliar insect herbivory are similar, including the production of jasmonates (Frost *et al.* 2008; Boeckler, Gershenzon & Unsicker 2013). It has not been tested yet, whether similar hormonal response mechanisms also occur in trees after herbivory by mammals.

The biosynthesis of secondary metabolites, such as phenolic compounds, can be stimulated through natural or simulated defoliation by insects (Roitto *et al.* 2009; Young *et al.* 2010), but also through browsing by mammals, be it natural (Bryant 1981; Gill 1992; Vourc'h *et al.* 2001) or simulated by clipping plant parts (Riipi *et al.* 2005). Yet, neither of these approaches can disentangle the effects of mechanical disturbance and chemical cues of the browsing animal.

An increasing number of studies demonstrate that plants are able to perceive elicitors specific to the feeding herbivore (Alborn *et al.* 1997; Halitschke *et al.* 2003; Schmelz *et al.* 2006; Schäfer *et al.* 2011b; Falk *et al.* 2014). For instance, the perception of elicitors from insect oral secretions can lead to elevated phytohormone production in the plant above the level induced by mechanical wounding (reviewed in Erb, Meldau & Howe 2012). However, salicylic acid, another plant hormone mainly involved in defence against pathogens, can inhibit these jasmonate-related defences (Gilardoni *et al.* 2011) and thereby compromise plant defence against herbivores. Although plants respond very specifically to insect salivary cues (Mithöfer & Boland 2012), only a few studies have examined whether trees respond equally specific to mammalian salivary cues. A study using moose saliva on sallow (*Salix caprea* L.) indicates that in addition to only tearing leaves, saliva application significantly increases the number of newly formed branches (Bergman 2002). Significant positive effects of saliva on shoot growth were also found using goat saliva on *Combretum apiculatum* Sonder (Combretaceae) in Botswana (Rooke 2003). Studying hormonal changes in trees after wounding and deer saliva application should help elucidate whether perception of deer-specific salivary elicitors has evolved in trees experiencing deer browsing. Analyses of defence metabolites should show whether hormonal responses have been translated into effective defence against this particular type of herbivory.

As the effectiveness of defence metabolites may vary between compound classes (Hagerman *et al.* 1992; Barbehenn & Constabel 2011), it is recommended to determine not only the total quantity but also the identity of the phenolics that are predominantly present or are induced by herbivore attack (Salminen & Karonen 2011). Hence, a comprehensive analysis of different groups of phenolics in trees and their changes after simulated browsing is desirable.

One distinctive characteristic of trees is their longevity, which makes individuals prone to browsing for several years, until they succeed to grow out of the reach of browsers, which in some cases takes up to 20 years (Seele 2011). During this time, apical buds are browsed repeatedly in winter, which can lead to detrimental growth delay (Gill 1992). However, apical bud removal often enhances cytokinin levels in the remaining lateral buds (Sachs & Thimann 1967; Kozlowski 1992), enabling trees to activate lateral bud meristems for regrowth and thus to tolerate browsing. Summer browsing mainly removes leaves, that is photosynthetically active material and carbohydrate reserves (Kozlowski 1992). Leaf removal often induces the production of defence metabolites, as was reported for some North American deciduous trees and boreal shrubs (Schultz 1988; Tuomi, Niemelä & Siren 1990). As nothing is known about responsiveness to salivary cues in buds compared to leaves, our research will consider differences in induced responses after browsing during both phenological stages to detect possible defence signals.

For our study, we chose sycamore maple (*Acer pseudoplatanus* L.) and European beech (*Fagus sylvatica* L.), as two dominant tree species of European mixed deciduous forests. The two species differ largely in their browsing intensity by roe deer, with maple being clearly preferred over beech (Seele 2011), as well as in their bud and leaf morphology. We simulated browsing by clipping apical buds or uppermost leaves of saplings of the two species growing within the reach of deer browsing and additionally applied roe deer saliva to the cut surface. In the remaining buds, or leaves, respectively, we studied immediate responses in herbivory-related phytohormones, that is jasmonates, salicylic acid and cytokinins, as well as subsequent changes in a broad variety of phenolics.

The following hypotheses were addressed:

1. Trees respond to deer browsing, simulated by clipping, with immediate changes in phytohormones in remaining buds and leaves. Deer saliva amplifies this response as compared to clipping alone.
2. The hormonal responses to simulated browsing lead to subsequent changes in defence metabolites in remaining buds and leaves. Deer saliva amplifies this response as compared to clipping alone.
3. Responses differ between buds and leaves. In buds, we mainly expect hormonal responses related to regrowth,

whereas changes in defence metabolites are expected to be more pronounced in leaves.

Materials and methods

STUDY SITE AND DESIGN

The study was conducted in the forest 'Leipziger Auwald' near the city of Leipzig in Saxony, Germany. The region has a continental climate with an average annual temperature of 8·88 °C and an average annual precipitation of 507 mm (German Meteorological Service (DWD)). The soil is a mosaic of Vega and Vega-Gleysol (Glaeser & Wulf 2009) formed by nutrient-rich fluvial clay. The forest canopy is dominated by pedunculate oak (*Quercus robur* L.) and European ash (*Fraxinus excelsior* L.), while the understorey regeneration mainly consists of maple species (*Acer pseudoplatanus* L., *A. platanoides* L.), European ash and, to a lesser extent, European beech (*Fagus sylvatica* L.).

We studied buds and leaves within the reach of roe deer of *Acer pseudoplatanus* L. and *Fagus sylvatica* L. (*Acer* and *Fagus* hereafter) with an average height of 54·3 (±13·1) cm and 68·8 (±13·9) cm, respectively. We only included saplings in our study which had not been browsed previously as evidenced by the absence of scars and growth deformations. Each sapling was randomly assigned to one of the following browsing treatments (Fig. 1a): (i) control, that is no treatment (n), (ii) clipping of apical buds or uppermost leaves (c) and (iii) clipping of apical buds or uppermost leaves and additional application of roe deer saliva to the cut surface (s). The amount of clipping was adjusted according to the size of buds and leaves to keep the impact on the sapling comparable, that is one main bud from *Acer*, and two smaller co-equal buds from *Fagus* were clipped, or two leaves from *Acer*, but four comparably smaller leaves from *Fagus* were clipped (Fig. 1b). For saliva application, we used samples of roe deer saliva from 24 adult (male and female) animals. Samples were taken as part of a larger study on life-history decisions and reproduction of roe deer at the field station of the Leibniz Institute for Zoo and Wildlife Research Berlin (IZW). Saliva samples were immediately deep frozen for storage and transport. For each the bud and the leaf study, 4–6 saliva samples were randomly chosen, defrosted in the field directly before use and pooled in order to minimize effects of different roe deer individuals and to obtain sufficient material. We applied 50 µL of saliva per cut using a pipette. To account for pure moisture effects, an equal amount of water was applied instead to the cut surface of individuals of the clipping treatment.

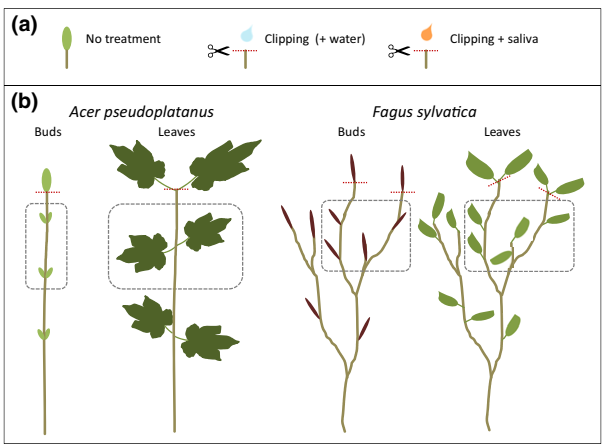

Fig. 1. Treatment design to study the effects of simulated deer browsing. (a) The first set of saplings was untreated and served as control. From a second set of saplings, apical buds or leaves were clipped and only water was applied to assess the effect of mere wounding. From a third set of saplings, apical buds or leaves were clipped and deer saliva was applied to the cut surface to assess deer-specific effects. (b) All three treatments were applied on both *Acer pseudoplatanus* and *Fagus sylvatica*, in the bud as well as in the leaf stage. Short dotted lines indicate cutting position. Systemic buds and leaves within dashed rectangles were harvested subsequently from all saplings to analyse induced changes in phytohormones and defence metabolites.

Buds/leaves underneath the clipped organs were harvested either 2 h or 2 days after treatment (see Fig. 1). Immediately after harvest, bud/leaf samples were frozen on dry ice and stored at −80 °C until extraction. Buds were treated and harvested shortly before budburst in mid-April 2013. Leaves were treated and harvested in late June and the beginning of July 2013. Five individuals were used for each plant species, phenological stage, treatment and time course, resulting in a total of 120 saplings.

CHEMICAL ANALYSES

Bud and leaf samples were prepared for analysis by grinding them with pestle and mortar in liquid nitrogen to a fine homogeneous powder. From buds of *Fagus,* we removed the non-leafy, husky bud scales and hairs before grinding to obtain a homogeneous powder and because these parts are not expected to show any physiological response. An aliquot of 50–100 mg of each sample was used for further analyses.

Analysis of phytohormones

The samples were extracted with 800 µL methanol containing isotopically labelled internal standards for jasmonic acid (D6-jasmonic acid, HPC Standards GmbH, Cunnersdorf, Germany), jasmonic acid-isoleucine (jasmonic acid-$13C_6$-isoleucine), salicylic acid (D4-salicylic acid, Sigma-Aldrich) and *trans*-zeatin (D5-*trans*-zeatin, OlChemIm, Czech Republic). Standards used for different phytohormones are given in Methods S1 (Supporting Information). Phytohormones were analysed by liquid chromatography-ESI-triple quadrupole mass spectrometry (LC-MS/MS) as described in Vadassery *et al.* (2012), with the modification, that an API 5000 mass spectrometer (Applied Biosystems, Darmstadt, Germany) was used. Cytokinins were analysed by LC-MS/MS as described by Schäfer *et al.* (2014).

Analysis of phenolics

Polyphenols were extracted with 1 mL of 80% methanol (methanol : water; 80 : 20; v : v) solution, and the resulting extract was diluted in a ratio of 1 : 10 (v : v) in water. Polyphenols in the diluted extracts were directly analysed by liquid chromatography with UV/diode array detection (LC-UV-DAD) to quantify abundant phenolics, as well as by LC-MS/MS to quantify pathway intermediates and compounds with lower abundance. For details on LC-UV-DAD of phenolic compounds, see Methods S2. Quantified phenolic compounds of each species' buds and leaves, respectively, were summed up according to major phenolic classes, that is phenolic acids, hydrolysable tannins, condensed tannins and flavonols. For details on LC-MS/MS of phenolic compounds, see Methods S3.

STATISTICAL ANALYSES

Differences in the concentration of phytohormones as well as phenolics between the three treatment levels were analysed using a

Kruskal–Wallis test. This nonparametric test allows the comparison of treatments similar to ANOVA, but without assuming a normal distribution. We used the function 'kruskalmc' in the package 'pgirmess' of the statistic software R, version 3.1.0 (R Core Team 2014), which accounts for multiple comparison among the three treatments (Siegel & Castellan 1988). Analyses were performed separately for buds and leaves of the two species as well as the two time steps.

Results

All phytohormones analysed and two out of four phenolic groups were responsive to simulated browsing, that is to clipping and/or saliva application. In many cases, saliva application either amplified or reversed the response observed after clipping alone. However, the intensity of the responses to clipping and to additional saliva application varied widely between phenological stages, that is buds and leaves, as well as between species.

HORMONAL RESPONSES

In the bud stage, clipping immediately induced significant changes in jasmonate levels in *Acer* but not in *Fagus* buds; especially, jasmonic acid (JA) and jasmonic acid-isoleucine (JA-Ile) levels increased in *Acer* buds to about three- to sevenfold, respectively, whereas hydroxy-jasmonic acid (OH-JA) levels did not change (Fig. 2a–c). In contrast to our hypothesis, additional saliva application did not amplify jasmonate signals in the buds of the two species. Surprisingly, neither clipping nor additional saliva application induced changes in salicylic acid (SA) levels in either of the species' buds (Fig. 2d).

In the leaf stage, the species' responsiveness was largely the reverse. Clipping induced significant changes in jasmonate levels in *Fagus* leaves, where JA and JA-Ile levels increased up to 10-fold, whereas in *Acer* leaves only OH-JA increased slightly after clipping (Fig. 2e–g). Additional saliva application did not amplify jasmonate responses in leaves, but tended to dampen the jasmonate-related signals. Interestingly, saliva application induced a threefold increase in SA levels in *Fagus* leaves compared to the control, but there was no response in *Acer* leaves (Fig. 2h).

Two days after the browsing treatments, almost no changes in the above-mentioned phytohormones were visible anymore in either of the species' buds or leaves, except a significant increase in OH-JA levels in *Fagus* buds (Fig. S1). Most likely, these hormonal responses only persisted for a short time.

Among the cytokinins detected in buds, trans-zeatin (tZ) and trans-zeatin riboside (tZR) were the most abundant in both tree species. tZ and tZR also belong to the most active cytokinins in many plant species (Stolz *et al.* 2011; Lomin *et al.* 2012; Shi & Rashotte 2012). Surprisingly, clipping induced an increase in tZ and tZR only in *Acer* buds, but clipping with saliva application induced a significant increase in tZ and tZR levels in *Fagus* buds compared to the control (Fig. 3). Data for cytokinins with lower abundances are shown in the Table S1. Cytokinin concentrations in leaves were below the detection limit and could thus not be further analysed.

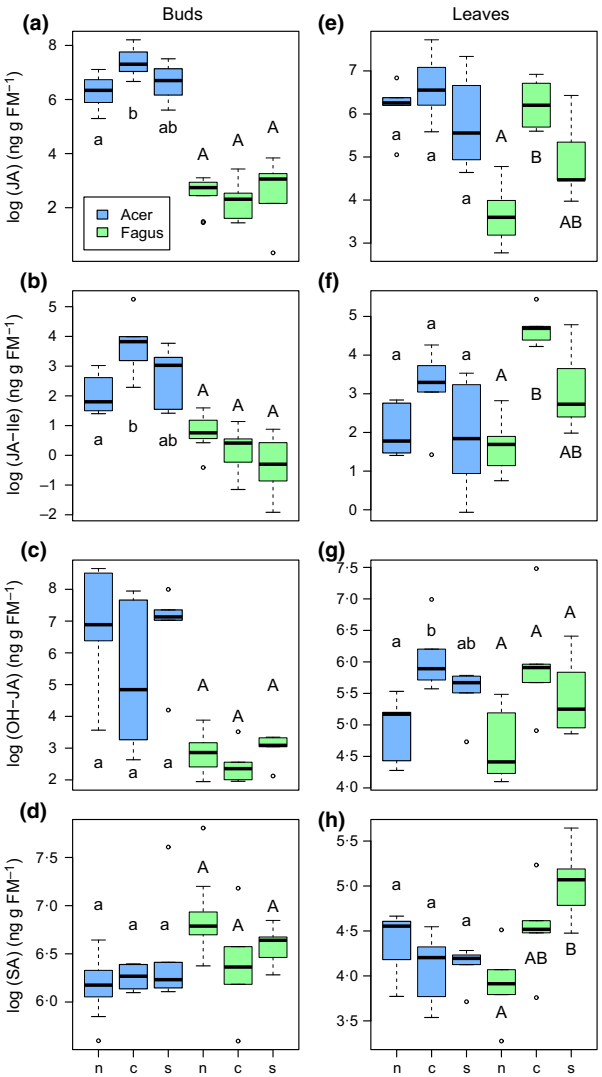

Fig. 2. Changes in bud and leaf phytohormones of *Acer pseudoplatanus* and *Fagus sylvatica* 2 h after simulated browsing. (a and e) JA – jasmonic acid; (b and f) JA-Ile – jasmonic acid-isoleucine; (c and g) OH-JA – hydroxy-jasmonic acid; (d and h) SA – salicylic acid. Treatments: n – none, c – clipping, s – saliva application in addition to clipping. Significant differences (multiple comparison Kruskal–Wallis test, $P < 0{\cdot}05$) between treatments within each species are indicated by different letters (*Acer pseudoplatanus* in lower case, *Fagus sylvatica* in upper case).

DEFENCE METABOLITES

The specific phenolic classes, as identified with LC-UV-DAD, and their responses to the different browsing treatments varied markedly between the two species as well as between phenological stages.

Fagus buds mainly contained phenolic acids (caffeoylquinates) and condensed tannin precursors (catechin

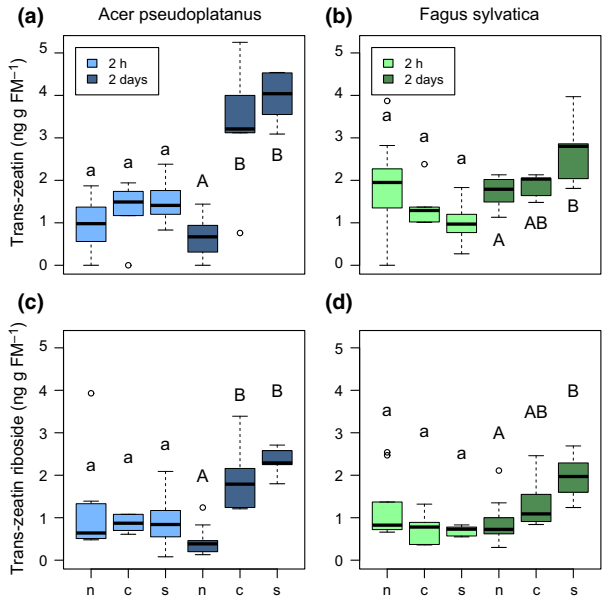

Fig. 3. Changes in bud cytokinins of *Acer pseudoplatanus* and *Fagus sylvatica* 2 h and 2 days after simulated browsing. (a, b) *trans*-zeatin, (c, d) *trans*-zeatin riboside. Treatments: n – none, c – clipping, s – saliva application in addition to clipping. Significant differences (multiple comparison Kruskal–Wallis test, $P < 0{\cdot}05$) between treatments within each time frame are indicated by different letters (2 h in lower case, 2 days in upper case).

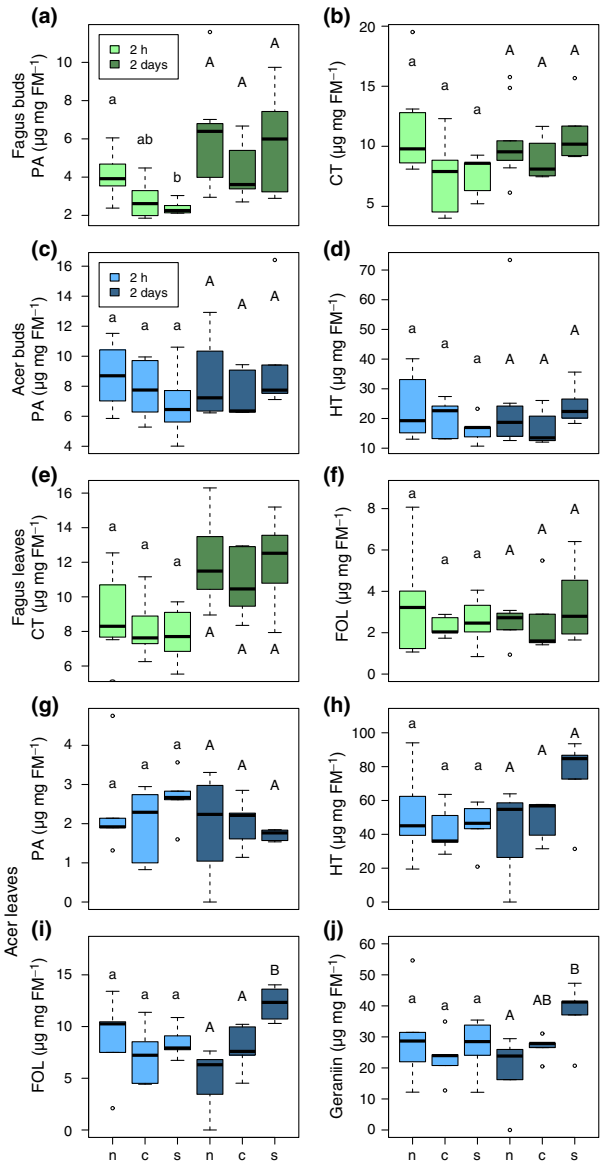

Fig. 4. Changes in bud and leaf phenolics of *Acer pseudoplatanus* and *Fagus sylvatica* 2 h and 2 days after simulated browsing. (a, b) *Fagus sylvatica* buds, (c, d) *Acer pseudoplatanus* buds, (e, f) *Fagus sylvatica* leaves, (g–j) *Acer pseudoplatanus* leaves. PA – phenolic acids, CT – condensed tannins, HT – hydrolysable tannins, FOL – flavonols; (j) geraniin isomers were the most abundant HT in *Acer pseudoplatanus* leaves and were also the most responsive. Treatments: n – none, c – clipping, s – saliva application in addition to clipping. Significant differences (multiple comparison Kruskal–Wallis test, $P < 0{\cdot}05$) between treatments within time frames are indicated by different letters (2 h in lower case, 2 days in upper case). Determination and calculation of phenolic group concentrations are based on LC-UV-DAD analyses.

and proanthocyanidin dimers and trimers), whereas *Acer* buds mainly contained phenolic acids (caffeic acid derivatives) and hydrolysable tannins (geraniin isomers; see Fig. S2 for chromatograms, and Table S2 for data on single phenolic compounds).

Changes in phenolic levels of buds after simulated browsing were mainly found in *Fagus*; more specifically, clipping plus saliva application led to a fast reduction in phenolic acid levels by about fifty per cent, compared to the control (Fig. 4a). Condensed tannin (CT) precursors did not change significantly (Fig. 4b). However, when looking at individual compounds within this large group, we did find a fast decrease in the monomer catechin after clipping of *Fagus* buds, which was followed by an increase in the dimer proanthocyanidin B1 after 2 days and additional saliva application (Table S2). Levels of phenolic acids and hydrolysable tannins (HT) in *Acer* buds did not change after clipping or saliva application (Fig. 4c,d).

Leaves of both species contained slightly different combinations of phenolics than buds. In *Fagus* leaves, we mainly found CT precursors (catechin, epicatechin, proanthocyanidin dimers and trimers) but also flavonols (quercetin glucoside, kaempferol glucosides and kaempferol rhamnoside), whereas in *Acer* leaves we found a wider range of phenolics, with phenolic acids (caffeic acid derivative and caffeoylquinates), HTs (maplexin, corilagin, geraniin isomers and some unknown ellagitannins) and flavonols (quercetin glucoside, quercetin rhamnoside, kaempferol rhamnoside and quercetin; see Fig. S3 for chromatograms and Table S2 for data on single phenolic compounds). The predominance of CT precursors in *Fagus* and of HTs in *Acer* conform to results from previous reports (Bate-Smith 1978; González-Hernández, Karchesy & Starkey 2003, respectively). No changes in phenolic levels after the browsing treatments were seen in *Fagus* leaves (Fig. 4e,f). In *Acer* leaves, phenolic acids and total

HTs remained unchanged (Fig. 4g,h), whereas when considering single phenolic compounds, clipping with saliva application induced a doubling in the concentration of the most abundant HTs in *Acer* leaves, geraniin isomers (Fig. 4j). Clipping with saliva application also led to a doubling of flavonol levels (Fig. 4i). The exact amounts of individual compounds as analysed with LC-UV-DAD as well as results for less abundant phenolics from LC-MS/MS are given in the Tables S2 and S3, respectively.

EFFECT OF DEER SALIVA

Application of deer saliva did not modify jasmonate responses in either *Acer* or *Fagus* buds or leaves (Fig. 2a–c and e,f), but did affect the saplings' responses with regard to other phytohormones and several defence metabolites. More specifically, concentrations of salicylic acid in *Fagus* leaves (Fig. 2h) as well as of cytokinins in *Fagus* buds (Fig. 3b,d) were only significantly raised, when the full treatment, that is clipping and saliva, was applied. Similarly, if significant changes in phenolics occurred, such as in *Fagus* buds and *Acer* leaves (Fig. 4a,i), it was only after clipping and saliva application, but not after mere wounding.

Discussion

Simulated deer browsing on *Acer* and *Fagus* saplings induced changes in phytohormones as well as in several classes of phenolics. Most strikingly, the application of deer saliva elicited different responses than clipping alone. Such changes in the chemical composition of temperate trees' buds and leaves in response to mammalian saliva have not been shown so far.

HORMONAL RESPONSES

Analysing hormonal responses has often been used to detect herbivore-specific perception in plants (Halitschke *et al.* 2001; Schmelz *et al.* 2009; Schäfer *et al.* 2011b). In our study, we found a general increase in jasmonate levels after clipping in both tree species, which is consistent with our expectations and with results from other plant species (e.g. Kallenbach *et al.* 2010; Schäfer *et al.* 2011b; Van-Doorn *et al.* 2011; Chauvin *et al.* 2013; Wang *et al.* 2013). Contrary to our hypothesis, deer saliva application did not further amplify jasmonate levels (Halitschke *et al.* 2001; Schmelz *et al.* 2009; Schäfer *et al.* 2011b), meaning that either no deer-specific elicitors exist that can trigger jasmonate biosynthesis in the two tree species studied or we missed the time window in which such changes might occur. Interestingly, in *Fagus* leaves, salicylic acid levels increased significantly after clipping and additional saliva application, while at the same time, jasmonate levels slightly decreased. Hence, there seems to be an antagonistic cross-reaction between jasmonates and salicylic acid. Whether deer saliva releases elicitors that trigger salicylic acid levels in trees to actively suppress jasmonate-mediated defences, as has been shown for other plant–herbivore interactions (Little *et al.* 2007; Diezel *et al.* 2009; Schäfer *et al.* 2011a; Kästner *et al.* 2014), awaits further investigation.

The two cytokinins *trans*-zeatin and its riboside, which were the most abundant in both *Acer* and *Fagus*, are also among the most active cytokinins with regard to the regulation of plant growth (Sakakibara 2006). Their increase following clipping of buds likely is a response to the removal of the apical dominance (Sachs & Thimann 1967) and will eventually lead to enhanced axillary shoot growth and enhanced branching, an effect that can be more detrimental to the sapling's performance than reduced growth (Gill 1992). As was expected and could be shown in this study, saliva can further amplify cytokinin levels and thus potentially enhance branching. This mechanism might explain earlier findings of increased branching of willow after a simulated browsing experiment with moose saliva (Bergman 2002). Increased cytokinin concentrations after herbivory are also suggested to play a pivotal role in redistributing the plant's primary and secondary metabolites (Giron *et al.* (2013). We analysed primary metabolites and found that fructose and glucose as well as several amino acids involved in the synthesis of phenolics, such as phenylalanine, tryptophan and tyrosine, decreased significantly in *Acer* buds, when deer saliva was applied (Methods S4, Tables S4 and S5, respectively). The observed decrease in primary metabolites likely reflects the increased investment into structural metabolites required for plant growth or into defence metabolites.

DEFENCE METABOLITES

Contrary to our expectations, none of the defence metabolites increased after clipping, but levels of some phenolic groups changed when deer saliva was applied additionally. Similar to conclusions from a meta-analysis on responses of woody species to insect damage (Nykänen & Koricheva 2004), we found that the responsive groups were mainly hydrolysable tannins (HT) and flavonols (both in *Acer*), whereas condensed tannins (CT), the dominating group in *Fagus*, did not change in response to browsing. This suggests that CT might rather act as a constitutive defence in *Fagus*. CTs have a high protein-binding capacity, thereby reducing protein digestibility in mammals (Barbehenn & Constabel 2011; Salminen & Karonen 2011). Although many browsers, especially deer, have tannin-binding salivary proteins (Austin *et al.* 1989), roe deer salivary proteins do not specifically bind to HT or CT (Gehrke 2005). A high amount of CT should thus deter browsing animals more effectively. Hagerman *et al.* (1992) suggested that CT interaction with proteins is positively associated with the tannin's molecular weight. Whether the decrease in CT monomers and subsequent increase in CT dimers in *Fagus* buds will be followed by a production of even higher polymeric CTs for a more

effective defence against browsing mammals could not be analysed in this study, but would require a longer time frame (Hammerbacher *et al.* 2014).

We also recorded increased levels of flavonols in *Acer* leaves after the full browsing treatment. Flavonols can affect growth rates of insect larvae (Mutikainen *et al.* 2000) and can also bind human proteins (e.g. Bi *et al.* 2004), but their role in defence against browsing mammals awaits further investigation.

Induced increases in single HTs, as we found in *Acer* leaves 2 days after clipping and saliva application, may not be sufficient as a defence against deer browsing. Feeding trials revealed that roe deer avoid pellets with high amounts of HTs, but prefer pellets with medium amounts over those without any tannins (Verheyden-Tixier & Duncan 2000). Thus, an increased HT content may only be an effective defence against roe deer browsing when concentrations reach a certain yet unknown threshold.

Most unexpected was the fast decrease in phenolic acids in *Fagus* buds within 2 h after clipping and saliva application. Phenolic acids are well-known pro-oxidants (Summers & Felton 1994) and might have undergone rapid oxidation upon simulated browsing, thereby releasing reactive oxygen species. As the effect of reactive oxygen species is gut pH dependent (Salminen & Karonen 2011), they likely do not act as defences against mammals but could potentially affect insect herbivores that are sensitive to oxidative stress. Moose browsing on silver birch (*Betula pendula* Roth.), for instance, reduces invertebrate herbivory levels, presumably through induced defence (den Herder *et al.* 2009).

How long the reported changes in phenolic levels of *Acer* and *Fagus* persist whether only for some days or for several years, as was found for mountain birch (*Betula pubescens* ssp. *tortuosa*) after simulated insect herbivory (Tuomi, Niemelä & Siren 1990), remains unknown. Our study, spanning a time frame from 2 h to 2 days, still covered a relevant portion of the time representative for temporal foraging patterns of roe deer, considering that roe deer use few distinct feeding sites within their home range, and may stay within high-quality patches for several hours, coming back to the patch several times during the day (Le Corre *et al.* 2008).

EFFECT OF DEER SALIVA

We hypothesized that saliva application would mostly amplify the responses already provoked by clipping. In fact, some responses were only significant with additional deer saliva application, such as increased levels of salicylic acid and of some defence metabolites. This response is probably due to the presence of some unknown elicitors in the saliva of mammalian herbivores. Compared to studies applying insect saliva to test plant responses (Turlings *et al.* 1993; Halitschke *et al.* 2001; Schäfer *et al.* 2011a, 2015), mammalian saliva has only been used in a very few cases (Bergman 2002; Rooke 2003; Tanentzap, Vicari & Bazely 2014). This is the first study to report hormonal responses together with changes in a broad range of defence metabolites after browsing of tree saplings grown under field conditions.

TREE RESPONSE TYPES – DIFFERENCES BETWEEN PHENOLOGICAL STAGES AND SPECIES

Based on their distinct functions in the course of the year, we hypothesized buds and leaves to respond to simulated browsing in different ways. Contrary to our expectations, phytohormones, especially jasmonates, did not only increase upon clipping in buds, which was the case for *Acer*, but increased also in *Fagus* leaves, and hence, an increase in jasmonates is likely a reaction to wounding, irrespective of the plant part, but specific to the species. Cytokinins increased more strongly in *Acer* than *Fagus* buds after clipping, which might be due to the strong monopodial growth of this species and the importance of a fast replacement of its main apical bud. Whether this hormonal response will translate in subsequent seasons into increased height growth enabling a faster escape from the reach of browsing animals, or into increased lateral branching, that is a spread of risk for single buds to be browsed, needs to be addressed in future studies. Both growth responses have been found in *Betula* species after winter browsing by moose (*Alces alces*; Danell, Bergstrom & Edenius 1994). We attribute the lower responsiveness of *Fagus* buds to their morphology, as they have a very pointed tip and are well protected by tough and hairy bud scales, making them structurally better defended than *Acer* buds.

The expectation that changes in defence metabolites are more pronounced in the leaf stage did not hold true for both species. Only in leaves of *Acer* did defence metabolites increase after simulated browsing. However, as stated earlier, the different types of phenolics might in this case be more important, as roe deer actively select food with small amounts of HT but avoid CT in feeding trials (Clauss *et al.* 2003), making *Fagus* with its high CT content in leaves the better defended species.

Both, the high structural defence of *Fagus* in the bud stage, and its high chemical defence in the leaf stage, are in agreement with the fact that *Fagus* is generally browsed less by roe deer and out-competes *Acer* saplings in areas of high browsing pressure (Seele 2011).

Concluding remarks

This study can be seen as a first attempt to translate measured chemical responses into response traits. Future studies could use a trait-based approach relating growth and defence responses to several browsing-relevant metabolic as well as mechanical traits across many tree species. This would help to reveal how species specific such responses

are or whether general patterns in response strategies of trees to mammalian browsing exist.

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Data accessibility

Data are available from the iBDP Biodiversity Data Portal, the institutional repository of the German Centre for Integrative Biodiversity Research (iDiv) Halle-Jena-Leipzig, Germany. The data can be accessed through https://idata.idiv.de/DDM/Data/ShowData/220.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Fig. S1. Changes in bud and leaf phytohormones 2 days after simulated browsing.

Fig. S2. LC-UV chromatograms showing most abundant defense metabolites in lateral buds.

Fig. S3. LC-UV chromatograms showing most abundant defense metabolites in subapical leaves.

Table S1. Cytokinin concentration (tZ, tZR, DHZR, IPR) in buds after simulated browsing.

Table S2. Concentration of major phenolic compounds after simulated browsing as analyzed by LC-UV-DAD chromatography.

Table S3. Relative quantification (peak area) of minor phenolic compounds (pathway intermediates and compounds with lower abundance) after simulated browsing as analyzed by LC-MS/MS chromatography.

Table S4. Soluble sugars in buds and leaves after simulated browsing.

Table S5. Free amino acids in buds and leaves after simulated browsing.

Methods S1. Internal standards used for the analysis of phytohormones.

Methods S2. LC-UV-DAD chromatography for analysis of abundant phenolics.

Methods S3. LC-MS/MS chromatography for analysis of minor phenolic compounds

Methods S4. Sugar and amino acid analyses.