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TRITROPHIC INTERACTIONS BETWEEN *POPULUS TREMULA*, LEAF BEETLES AND THEIR NATURAL ENEMIES - FROM THE FIELD TO THE LABORATORY

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GENERAL INTRODUCTION:
TRITROPHIC INTERACTIONS BETWEEN *POPULUS TREMULA*, LEAF BEETLES AND THEIR NATURAL ENEMIES - FROM THE FIELD TO THE LABORATORY

INTRODUCTION

A drastic increase of global energy consumption is expected for the following years. The global goal (Kyoto protocol) is to replace fossil fuel by renewable energy sources (Lasch et al., 2010), thus the cultivation of renewable energy gains in importance, e.g. with trees as a living store of biomass (Hinchee et al., 2009). In this context short rotation plantations with fast-growing tree species and rotation times of less than 30 years (Makeschin, 1999) got increasingly into focus of current ecology research but have been already aresearch subject of applied sciences since the 1970s. Short rotation cropping systems can help to meet the requirements of future wood demands and can also sequester carbon as a contribution to the reduction of greenhouse gases (Dickmann, 2006). However, today these cropping systems are not able to supply the actual wood demand, as the plantations provide only 12% of the total amount of wood consumed, whereas the rest of 88% is still taken from wild forests (Fenning and Gershenzon, 2002). The Food and Agriculture Organization of the United Nations (FAO) expected an increase of demand for wood from today 1.7% annually up to 20 % in the following decades. To meet these requirements 9.4 million hectares of forest cover will be destroyed every year (FAO, 2008). The exploitation of native forests at current rates will lead to a massive depletion of this resource. Moreover, the destruction of native forests will result in an irretrievable loss of habitats and in the endangering of wildlife (Fox, 2000, Fenning and Gershenzon, 2002). Therefore, high-yielding short rotation cropping systems on former arable land are a worthwile alternative to the exploitation of natural habitats.

Poplar short rotation forestry

In short rotation plantations mainly *Salix* and *Populus* species are cultivated which exhibit economically important characteristics such as fast growth-rates (Zsuffa et al., 1996), efficient nitrogen storage (Pregitzer et al., 1990), high photosynthetic capacity (Barigah et al., 1994), high tolerance and resistance against damage by herbivores (Bassman et al., 1982), and global propagation (Yu, 2001, Dickmann and Kuzovkina, 2008). Such tree species are commonly used to obtain pulp, chipboard and energy (Bradshaw et al., 2000).

Additionally, *Populus* species have become a favorite subject in ecology conservation, environmental sciences, molecular studies – with the first completely sequenced tree genome

(Tuskan et al., 2006) –, physiology and biotechnology (Bradshaw et al., 2000) due to their important role in several ecosystems. *Populus* species provide habitat for wildlife (Sage, 1998), act as keystone species with a high level of genetic diversity at the population level (David et al., 2001), and are involved in complex community level interactions. For example Madritch et al. (2007, 2009) showed the linkage between genetic diversity and variation in belowground processes in trembling aspen, and Whitham et al. (2006) discussed the importance of genetic diversity in evolutionary processes in natural forests.

Poplars are pioneer species and common plant invaders in the early succession on disturbed sites. They are widespread trees species in boreal, temperate, montane as well as tropical forests (Dickmann and Kuzovkina, 2008) and include about 30 species in the northern hemisphere (Eckenwalder, 1996). In natural sites aspen create habitats for a fauna including many endangered species and therefore provide important ecosystem services (Kouki et al., 2004).

Agricultural and short rotation forestry production is focused on maximum yield which is achieved by extended cultures of selectively breed, but ecologically less adapted crop and tree varieties. This type of management causes the instability and pest susceptibility of such cropping systems (Gruppe et al., 1999). In willow plantations the blue willow leaf beetle *Phratora vulgatissima* prefers trees cropped in monoculture (Peacock et al., 1999). With regard to cropping systems two principal hypotheses are known as drivers for lower herbivore densities: 1) the “enemies hypothesis”: predators and parasites are more effective in complex systems than in simple ones; and 2) the “resource concentration hypothesis”: specialist herbivores are able to find, stay, and reproduce more easily in simple systems, i.e. monocultures of their host plants (Root, 1973, Risch, 1981, Russell, 1989). Lacking natural resistance against pathogens and insect pests is compensated by intensive use of pesticides (Gruppe et al., 1999). The application of insecticides in short rotation plantations is reported to increase the biomass growth (Gruppe et al., 1999). However, Bassman et al. (1982) estimated that defoliation of poplars up to 75% results in only a negligible negative growth impact. A reduction of feeding damage by herbivores can be achieved by the diversification of plant cropping (Stamps and Linit, 1998). Planting of diverse poplar and willow species together in a plantation can reduce the feeding damage by herbivorous insects due to the fact that diversity in cropping systems can alter the behaviour of herbivorous invertebrates (Vandermeer, 1989).

Multitrophic interactions

A huge variety of abiotic and biotic factors affect the abundance of herbivores and the impact of natural enemies, e.g. fluctuations in weather or availability and quality of food. Thus, plants (as the food resource) exert a bottom-up influence on herbivorous insects and their natural enemies by their distribution, availability, their architecture and by primary as well as secondary metabolites (Ehrlich and Raven, 1964, Price et al., 1980, Bottrell et al., 1998, Bernays and Chapman, 2000, Legrand and Barbosa, 2003, Schoonhoven et al., 2005). There are direct and indirect ways of such bottom-up influences of plants. Plant morphological traits including plant size (Neuvonen and Niemelä, 1981, Lawton, 1983), plant biomass (resource abundance) (Hunter, 1992, Marques et al., 2000), leaf surface characteristics such as pubescence, aspects of plant color and shape and phenological differences as well as plant allelochemicals act directly as well as indirectly (Kagata et al., 2005) on herbivores and natural enemies. Also, plant population traits such as variation in plant density and plant diversity have a direct impact on arthropod communities. Indirect effects are mainly driven by infochemicals, e.g. herbivore-induced plant volatiles (HIPVs) (Bottrell et al., 1998, Dicke and Baldwin, 2010). Multitrophic interactions are often shaped by such herbivore-induced plant volatiles (HIPVs) released by infested plants, as well as by herbivore sequestered plant allelochemicals, e.g. salicyladehyde sequestered by salicin using leaf beetle larvae, and by prey volatiles such as pheromones and de novo synthesized allomones (Fig. 1).

Infochemical use is known for a range of herbivores (Rojas, 1999, Kalberer et al., 2001, Halischke et al., 2008) and of the herbivores' enemies (Takabayashi and Dicke, 1996, Steidle and van Loon, 2003, Dicke and Baldwin, 2010) including parasites (Dougherty et al., 1999), parasitoids (Rutledge, 1996, Quicke, 1997) and predators including beetles (Herms et al., 1991, Yoneya et al., 2009), true bugs (Weissbecker et al., 2000) and wasps (Hendrichs et al., 1994, Punzo and Ludwig, 2005). These infochemicals are used for host finding and location and thus link two trophic levels, the first and second level (plant – herbivore) as well as the second and third level (prey – predator) (Fig. 1).

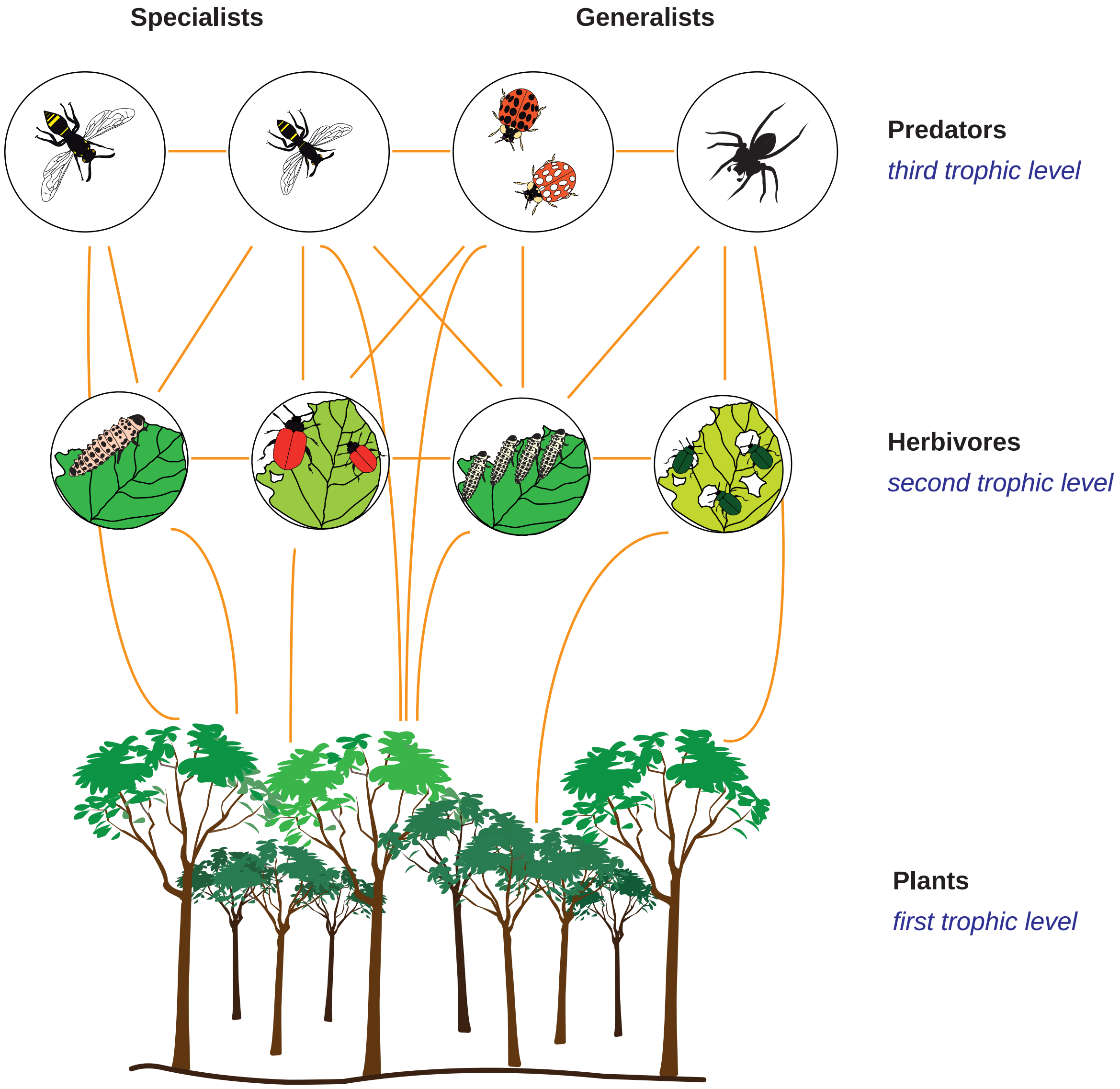


Figure 1: Scheme of multitrophic interactions modified after Dicke and Baldwin (2010). Orange lines represent infochemically mediated interactions.

Previous research in community ecology is dominated by investigating such two trophic level interactions (plant-herbivore and predator-prey) (Tscharntke and Hawkins, 2002). Studies dealing with the tritrophic system consisting of *Populus* and/or *Salix*, herbivorous insects, and their natural enemies were also often concentrated on only two levels.

In the last three decades many studies have been conducted regarding the interactions of the first and second trophic level, between willows or poplars and different herbivorous insects, especially specialized leaf beetles of the genera *Chrysomela* (Augustin et al., 1993, La Spina et al., 2010) and *Phratora* (Finet and Gregoire, 1982, Peacock and Herrick, 2000, Peacock et al., 2004). Genetic differences of the tree species were found to drive tolerance and resistance against herbivore damage (Denno et al., 1990, Shen and Bach, 1997). A lot of studies refer to the leaf chemistry and the relationship with phytophagous insects (Osier and Lindroth, 2006, Donaldson and Lindroth, 2007, Stevens et al., 2007). Additionally, several studies were conducted on the interactions between specialized salicin using leaf beetle larvae and natural enemies (specialists and generalists) (Pasteels and Gregoire, 1984, Smiley, 1991, Gathmann and Tscharntke, 1999, Gross et al., 2004), but less is known about multitrophic interactions in this system. Only few studies investigated tritrophic interactions on willows: Sipura (1999) showed that bird predation can alter the patterns of insect densities on willows with positive effects for the trees. Kagata et al. (2005) described indirect bottom-up effects in laboratory experiments of leaf beetle larvae to a lady bird beetle in a system with cut and uncut willows. Cha et al. (2009) proved that the preference of *Chrysomela knabi* for phenolic glycoside rich willow plants increases larval growth and survival, and that predation pressure by common and exotic generalist predators would reinforce this preference.

STUDY ORGANISMS AND DESIGN

The Göttingen Poplar Diversity Project

The Göttingen Poplar Diversity Project is a sub-project of the Göttingen cluster of excellence “Functional Biodiversity Research”, which was established in 2008 at the University of Göttingen. The overall topic of the cluster is biodiversity and ecosystem functioning, including experiments in grasslands and historical studies in terms of long-term biodiversity change. The poplar diversity project involves several working groups investigating the functional role of intraspecific diversity in woody plants using aspen (*Populus tremula*) as a model species. The molecular analyses of the *P. tremula* trees were performed by the Department of Forest Genetics and Forest Tree Breeding. The Department of Plant Ecology and Ecosystem Research and the Department of Forest Botany and Tree Physiology adressed the questions how phenological, morphological and physiological traits as well as plant-fungi interactions affect growth performance of the trees. The Department of Forest Zoology and Forest

Conservation investigated the Volatile Organic Compound (VOC) emission by the trees and the influence of VOCs on herbivorous insects. My own thesis aimed at analyzing the influence of different morphological and chemical tree traits (leaf chemistry and herbivore-induced plant volatiles) on herbivorous insects and their natural arthropod enemies.

Study organisms – The Populus tremula *trees*

The European aspen (*Populus tremula*) was used as model tree for the experiments, because this economically impacted species is able to reach considerable growth rates even on poor soils and under unfavourable habitat conditions (Hofman, 1998) and is less sensitive to drought like e.g. *P. trichocarpa*. The European aspen is a pioneer species with an extensive distribution range and is one of the most widespread tree species in boreal and temperate forests (Bradshaw et al., 2000, Dickmann and Kuzovkina, 2008). Aspen reproduce via root suckers as well as via sexual reproduction and evolved several aspen genotypes caused by lots of possible crossing constellations of parent trees in given surroundings. This high level of genetic diversity creates genetically rich tree patches (Madritch et al., 2007, 2009). Within these patches Madritch et al. (2009) found that ecosystem soil processes are linked with canopy herbivore interactions. Naturally grown aspen forests provide habitat and food for numerous insect and pathogen species, even endangered ones (David et al., 2001, Kouki et al., 2004), and they are significantly embedded in the carbon cycle by their high rates of carbon sequestration (Kurz and Apps, 1999). Hence, aspen became more important in short rotation forestry because of its ecological functions (Kouki et al., 2004) as well as a possible resource for biomass production on marginal soils (Hofman, 1998). Because of this benefit aspen may be preferred to drought sensitive species such as *P. trichocarpa* under predicted future climate scenarios with increasing drought periods in summer.

The *P. tremula* trees used in our experiments originated from eight full-sib families with German origin. The progenies of each full-sib family were bred by controlled crossings in 2000, from parent tree material originating from 30-year-old trees selected in the district of Geismar in Göttingen. The crossings and molecular analyses of the trees were conducted by the Department of Forest Genetics and Forest Tree Breeding.

Study organisms – Leaf beetles and natural enemies (arthropods)

The leaf beetles of the genera *Chrysomela*, especially *C. populi* and *C. tremulae*, and *Phratora* (*Phratora vitellinae* and *P. vulgatissima*) are reported to be the most important defoliating

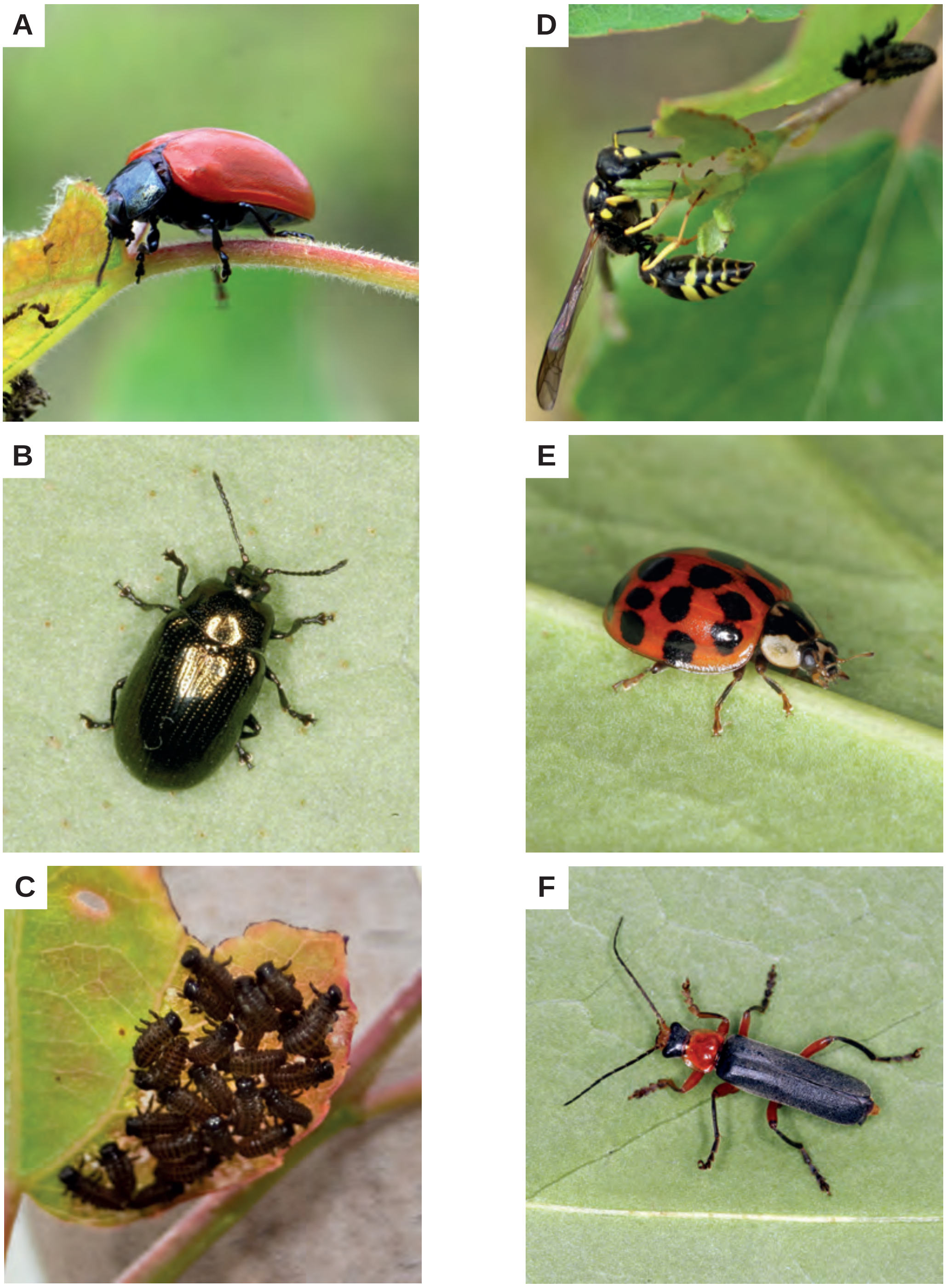


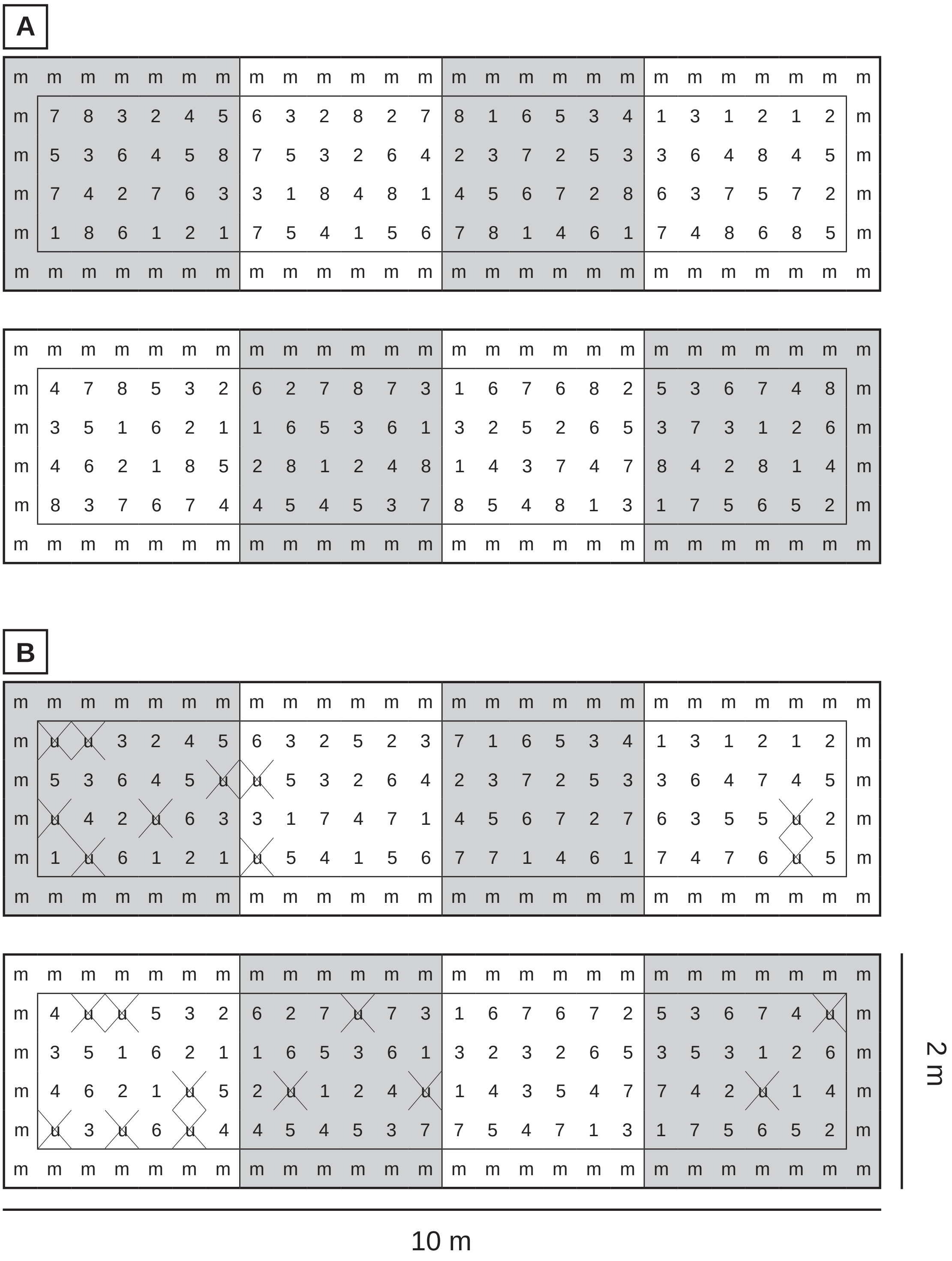
Figure 2: Pictures of leaf beetles, their larvae (**A**-**C**) as well as specialist and generalist predators (**D**-**E**). **A**: *Chrysomela populi*. **B**: *Phratora vitellinae*. **C**: *Phratora* sp. larvae. **D**: *Symmorphus gracilis*. **E**: *Harmonia axyridis*. **F**: *Cantharis pellucida*. **B**, **E** and **F** copyright by Christoph Benisch (www.kerbtier.de).

pests in young short rotation plantations in Central Europe (Sinreich, 1955, Augustin et al., 1993, Gruppe et al., 1999). Among these leaf beetle species such as *Phratora vulgatissima* and *Galerucella lineola* prefer leaves with low concentrations of phenolic glycosides (Kendall et al., 1996, Orians et al., 1997, Glynn et al., 2004), whereas *Chrysomela populi, Phratora vitellinae* and *Gonioctena decemnotata,* prefer salicaceous tree species with relatively high concentrations of phenolic glycosides in the leaves (Rowell-Rahier, 1984, Orians et al., 1997, Ikonen, 2002). Leaf beetle larvae feeding on willow and poplar sequester phenolic glycosides, especially salicin and its derivatives, to salicylaldehyde (Pasteels et al., 1983, Smiley et al., 1985, Köpf et al., 1998). This salicylaldehyde provides protection against several generalist arthropod predators such as ants (Wallace and Blum, 1969), ladybird beetles (Smiley, 1991) and spiders (Palokangas and Neuvonen, 1992) (Fig. 2). Only specialized natural enemies such as eumenid wasps (Blüthgen, 1961, Gathmann and Tscharntke, 1999), phorid flies (Zvereva and Rank, 2004), syrphid flies (Rank and Smiley, 1994, Gross et al., 2004) and the sawfly *Tenthredo olivacea* (Pasteels and Gregoire, 1984) are reported to attack the leaf beetle larvae because they are attracted by the larvae's defense secretion.

Experimental sites – Common garden experiments

Two common-garden experiments with *P. tremula* were established in 2008. One was established in beds next to the greenhouses of the Department of Forest Botany and Tree Physiology at the University of Göttingen (a); the other experiment was established on a study site next to the greenhouse of the Institute of Agroecology (b). Both sites are located in Göttingen. In both experiments *P. tremula* trees of eight full-sib families were selected and planted. For the first experiment (a) trees were planted in a randomized design in two blocks. Each block contained four plots and each plot included three aspen saplings of each full-sib family (Fig. 3). The trees were planted with a distance of 40 cm to each other. An additional treatment with a systematic fungicide was applied monthly on four plots, whereas the other four plots were treated with water and acted as controls.

For the second experiment (b) trees were planted in a randomized block design containing nine plots (Fig. 4 A). Each full-sib family was represented by 18 trees. The trees were planted into pots with a distance of 2 m between two pots following the randomized design with 144 trees. The trees were watered as necessary in both experiments.



A

m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m
m	7	8	3	2	4	5	6	3	2	8	2	7	8	1	6	5	3	4	1	3	1	2	1	2	m
m	5	3	6	4	5	8	7	5	3	2	6	4	2	3	7	2	5	3	3	6	4	8	4	5	m
m	7	4	2	7	6	3	3	1	8	4	8	1	4	5	6	7	2	8	6	3	7	5	7	2	m
m	1	8	6	1	2	1	7	5	4	1	5	6	7	8	1	4	6	1	7	4	8	6	8	5	m
m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m

m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m
m	4	7	8	5	3	2	6	2	7	8	7	3	1	6	7	6	8	2	5	3	6	7	4	8	m
m	3	5	1	6	2	1	1	6	5	3	6	1	3	2	5	2	6	5	3	7	3	1	2	6	m
m	4	6	2	1	8	5	2	8	1	2	4	8	1	4	3	7	4	7	8	4	2	8	1	4	m
m	8	3	7	6	7	4	4	5	4	5	3	7	8	5	4	8	1	3	1	7	5	6	5	2	m
m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m

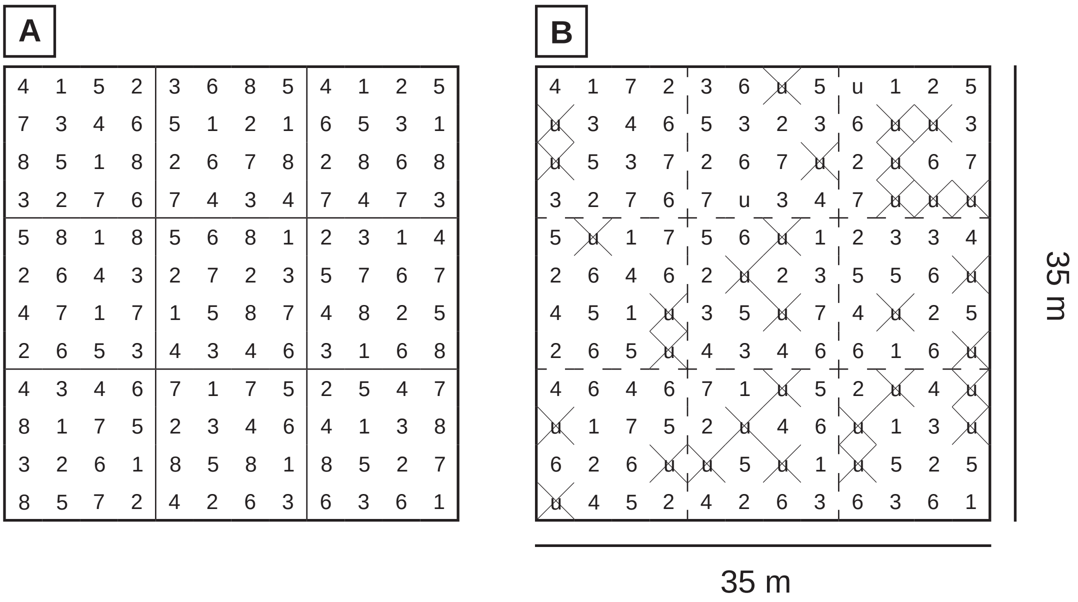
B

m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m
m	~~u~~	~~u~~	3	2	4	5	6	3	2	5	2	3	7	1	6	5	3	4	1	3	1	2	1	2	m
m	5	3	6	4	5	~~u~~	~~u~~	5	3	2	6	4	2	3	7	2	5	3	3	6	4	7	4	5	m
m	~~u~~	4	2	~~u~~	6	3	3	1	7	4	7	1	4	5	6	7	2	7	6	3	5	5	~~u~~	2	m
m	1	~~u~~	6	1	2	1	~~u~~	5	4	1	5	6	7	7	1	4	6	1	7	4	7	6	~~u~~	5	m
m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m

m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m
m	4	~~u~~	~~u~~	5	3	2	6	2	7	~~u~~	7	3	1	6	7	6	7	2	5	3	6	7	4	~~u~~	m
m	3	5	1	6	2	1	1	6	5	3	6	1	3	2	3	2	6	5	3	5	3	1	2	6	m
m	4	6	2	1	~~u~~	5	2	~~u~~	1	2	4	~~u~~	1	4	3	5	4	7	7	4	2	~~u~~	1	4	m
m	~~u~~	3	~~u~~	6	~~u~~	4	4	5	4	5	3	7	7	5	4	7	1	3	1	7	5	6	5	2	m
m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m	m

Figure 3: Design of the poplar common-garden experiment at the Department of Forest Botany and Tree Physiology at the University of Göttingen (**A**) before and (**B**) after the additional molecular analyses of the trees. 24 progenies of eight (1-8) full-sib families were planted in eight plots, of which four (grey) were treated monthly with a systematic fungicide and four were used as control. m = margin trees (*P. tremula*). u = tree of unknown full-sib family.

An additional molecular analysis of the trees of all full-sib families revealed that the trees of full-sib family C8 (in both experiments) had to be excluded from statistical analyses because they cannot assign to an own full-sib family. Due to this fact the block design of experiment (b) had to be revised into a fully randomized design (Fig. 4 B).



4	1	5	2	3	6	8	5	4	1	2	5
7	3	4	6	5	1	2	1	6	5	3	1
8	5	1	8	2	6	7	8	2	8	6	8
3	2	7	6	7	4	3	4	7	4	7	3
5	8	1	8	5	6	8	1	2	3	1	4
2	6	4	3	2	7	2	3	5	7	6	7
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2	6	5	3	4	3	4	6	3	1	6	8
4	3	4	6	7	1	7	5	2	5	4	7
8	1	7	5	2	3	4	6	4	1	3	8
3	2	6	1	8	5	8	1	8	5	2	7
8	5	7	2	4	2	6	3	6	3	6	1

4	1	7	2	3	6	~~u~~	5	u	1	2	5
~~u~~	3	4	6	5	3	2	3	6	~~u~~	~~u~~	3
~~u~~	5	3	7	2	6	7	~~u~~	2	~~u~~	6	7
3	2	7	6	7	u	3	4	7	~~u~~	~~u~~	~~u~~
5	~~u~~	1	7	5	6	~~u~~	1	2	3	3	4
2	6	4	6	2	~~u~~	2	3	5	5	6	~~u~~
4	5	1	~~u~~	3	5	~~u~~	7	4	~~u~~	2	5
2	6	5	~~u~~	4	3	4	6	6	1	6	~~u~~
4	6	4	6	7	1	~~u~~	5	2	~~u~~	4	~~u~~
~~u~~	1	7	5	2	~~u~~	4	6	~~u~~	1	3	~~u~~
6	2	6	~~u~~	~~u~~	5	~~u~~	1	~~u~~	5	2	5
~~u~~	4	5	2	4	2	6	3	6	3	6	1

Figure 4: Design of the poplar common-garden experiment at the greenhouse area of the Agroecology Institute at the University of Göttingen (**A**) before and (**B**) after the additional molecular analyses of the trees. 18 progenies of eight (1-8) full-sib families were planted into pots with two meter distance between two pots. Dashed line = altered block design. u = tree of unknown full-sib family.

Experimental sites – Chemical field bioassays

The field bioassays were performed testing the leaf beetle larvae's allomone, salicylaldehyde, in 2009 and 2010 on an area with an young seral stage in the vicinity of Göttingen near Lutterberg (51°37´N 9°64´O). The field is located under a high-tension line, the reason for which the trees are cut at regular intervals.

RESEARCH OBJECTIVES AND CHAPTER OUTLINE

In the present study we investigated the effects of morphological and chemical (leaf chemistry) traits of genetically closely related aspen (*P. tremula*) on herbivorous insects. The general focus of this study was to characterize trait variability in aspen differing in genetic relatedness, with an emphasis on productivity (chapter 2). We analyzed the influence of morphological traits as well as the effects of herbivore-induced plant volatiles (HIPV) on specialized leaf beetle adults and larvae and predatory arthropods over two generations of the leaf beetles (chapter 3). Further focal points were the odor perception of HIPVs and salicylaldehyde (the beetle larvae's allomone) of specialized predators of salicin using leaf beetles. We investigated three potter wasp species differing in their prey specialization (chapter 4). Finally we tested the attractiveness of salicylaldehyde to generalist predatory beetles such as *Harmonia axyridis* (Coccinellidae) and *Cantharis pellucida* (Cantharidae) (chapter 5).

Our main hypotheses were (Fig. 4):

I. Variability of morphological, phenological and chemical traits differs between aspen along a genetic gradient (chapter 2 & 3).
II. Chemical and genetic traits influence biomass productivity and herbivore abundances (chapter 2).
III. Plant size and resource abundance, e.g. leaf number, as well as herbivore-induced plant volatiles influence specialized leaf beetles and their natural enemies (chapter 3).
IV. Generalist predators are able to detect defense compound of salicin using leaf beetle larvae (salicylaldehyde) (chapter 4).
V. Specialist predatory wasps are able to perceive volatiles emitted by prey and the prey's host plants (chapter 5).

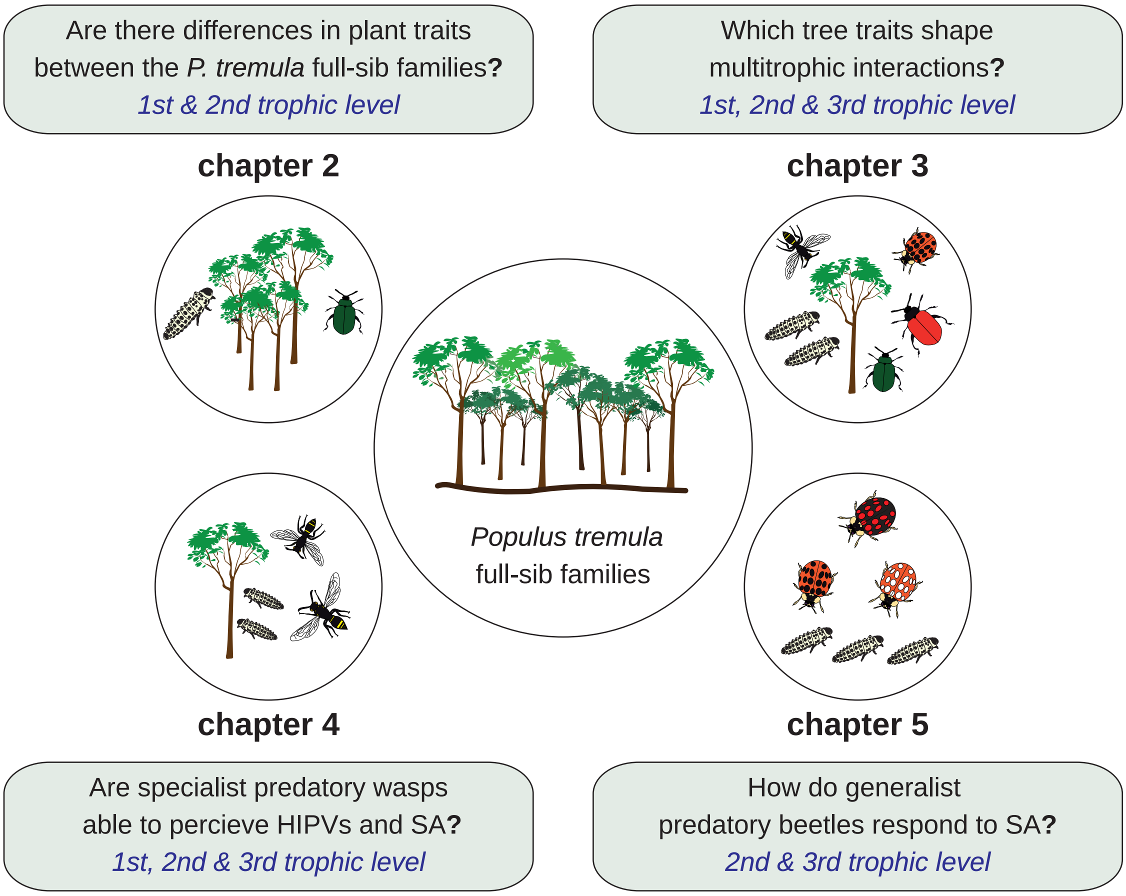

Figure 5: Scheme of the study objectives with the main questions of each chapter of the thesis. HIPVs = herbivore-induced plant volatiles. SA = salicylaldehyde.

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CHAPTER

2

RELATING GENETIC VARIATION OF ECOLOGICALLY IMPORTANT TREE TRAITS TO ASSOCIATED ORGANISMS IN FULL-SIB ASPEN FAMILIES

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ABSTRACT

Knowledge on phenological, morphometric, and phytochemical variation of local progenies of European aspen (*Populus tremula*) is limited. The goal of this study was to characterize variation in growth and ecologically important leaf properties in aspen full-sib families in relation to interacting organisms (mycorrhiza, endophytes and insects) and to determine if these interactions were affected by soil-application of a systemic fungicide. In local progenies, within family variation of neutral molecular genetic markers (nuclear microsatellites) was higher than between families. Significant variation in growth, production of phenolic defensive compounds and other phytochemical leaf traits was found between families. Phenolic compounds showed clear negative correlation with generalist herbivores, but did not result in negative trade-off with biomass production. Differences in mycorrhizal colonization were not found among full-sib families and application of a systemic fungicide suppressed neither mycorrhizal colonization nor infestation with insects. However, a strong suppression of endophytes occurred, whose long-term consequences may require attention when fungicides are used in agro-forestry plantations.

Key Words: Agro-forestry, molecular marker, nitrogen, nutrition, phenolic compounds, *Populus*

INTRODUCTION

European and American aspen (*Populus tremula*, *P. tremuloides*) are among the most wide-spread tree species in circumpolar boreal and temperate forest regions (Hultén and Fries, 1986, Dickmann and Kuzovkina, 2008). They are pioneering species with low nutrient demand that colonize disturbed and shallow soils (Dimpflmeier, 1963, Tamm, 2006). In contrast to other poplar species that are typically found in alluvial, riparian and wetland ecosystems, aspens are relatively drought tolerant and form distinct forest communities. In past European silvicultural practices aspens have usually been removed to avoid competition in even aged, traditionally managed coniferous forests (DeChantal et al., 2009). However, it has recently been recognized that aspen create habitats for specific fauna including many endangered species and therefore provide important ecosystem services (Kouki et al., 2004). To date, aspens are increasingly valued because of their ecological functions as well as a possible resource for biomass production on marginal soils.

Traits of ecological and economic value have mainly been studied in American aspen (*P. tremuloides*) or in hybrids of *P. tremuloides* with *P. tremula* (Li and Wu, 1997, Liesebach et al., 1999), whereas less information is available on its close relative, European aspen. American aspen show significant genetic variation in the phytochemistry of defence compounds such as phenolic glucosides and condensed tannins, whereas other foliar traits such as nitrogen content respond mainly to environment (Osier and Lindroth, 2006, Donaldson and Lindroth, 2007). Phenolic compounds protect against *Venturia* shoot blight infection (Holeski et al., 2009) and influence feeding behaviour of herbivorous insects on Salicaceae (Rowell-Rahier, 1984, Donaldson & Lindroth, 2007). Among herbivores, generalists such as the chrysomelid beetles *Phratora vitellinae*, *Phratora vulgatissima* and *Galerucella lineola* prefer leaves with low concentrations of phenol glucosides (Kendall et al., 1996, Orians et al., 1997, Glynn et al., 2004). In contrast, specialized chrysomelid beetles, for example, *Chrysomela populi* and *Gonioctena decemnotata*, prefer salicaceous species with relatively high concentrations of phenol glycosides in the leaves (Orians et al., 1997, Ikonen, 2002) because their larvae sequester plant-derived allelochemicals such as salicylaldehyde for defence (Pasteels et al., 1983).

Trade-off between allocation to defence compounds and growth has been reported, at least under limiting nutrient resources (Donaldson et al., 2006). Therefore, differences in growth and biomass production among *P. tremula* progenies may have consequences for phytochemical traits, in particular for allocation of defence compounds, and for interactions with associated organisms such as mycorrhizae, endophytes or leaf feeding insects. Since economically and ecologically sustainable biomass production requires cultivation of trees adapted to regional climate with optimum growth and stress tolerance, we investigated variation in phenological, morphological and ecophysiological traits in a common garden experiment with seven full-sib families generated by crossing of local *P. tremula* parent trees (Göttingen, Central Germany). It is often required to apply phyto-protective agents to prevent spreading of diseases in plantations. Therefore, we also studied the influence of a systemic fungicide on plant performance and biotic interactions. The following hypotheses were tested: (i) fullsib aspen families show significant variation in growth and ecophysiological leaf traits that are related to parenthood and modulate interactions with associated organisms; (ii) defensive compounds and growth are negatively related providing evidence for an energetic trade-off; (iii) application of a systemic fungicide has negative effects on associated organisms such as endophytes, mycorrhiza, and leaf-feeding insects.

MATERIAL AND METHODS

Plant materials and experimental set-up

The parent *P. tremula* trees were located close to Göttingen (Geismar, 51° 31′ N, 9° 57′E). In the year 2000, shoots with male and female flowers were transported to a greenhouse and used for controlled crossing of male trees number 1, 3 and 5 with female trees number 2, 4, 7, 8, and 9, respectively, resulting in the following crossings: C1 (4x5), C2 (9x5), C3 (8x5), C4 (2x5), C5 (2x3), C6 (9x3), and C7 (7x1). Seeds were germinated on moist Vermiculite (grain size 3 to 8 mm, Deutsche Vermiculite Dämmstoff GmbH, Sprockhövel, Germany). Seedlings were planted in pots (Fruhsdorfer soil, type N, Fruhsdorf, Germany), cultivated outdoors and watered as necessary. In spring 2008, 8-yrs-old trees were out-planted according to a randomized block design with 8 blocks (4 blocks treated monthly with 75 l Amistar

Opti [25µl L-1, Syngenta, Maintal, Germany], 4 control blocks treated with water). Each block contained 24 plants; i.e. 3 trees of each of the 7 full-sib families in addition to 3 plants of a further crossing which was however contaminated and therefore not included in further analyses. A bed contained four blocks of alternating control and fungizide treated blocks, which were separated by plastic barriers and surrounded by a row of additional trees to avoid edge effects. The experiment consisted of two beds. The trees were cultivated for one growth phase and watered as necessary. The mean ambient air temperature was 15°C.

Phenological and morphometric measurements

Before bud break, diameter at the bottom (root collar) and height of the main shoot of each tree were measured. Bud break at the apex of the leader shoot was scored regularly. The Julian days were recorded until the first leaf was fully expanded (according to the scores described by UPOV, 1981). The trees were harvested in the first week of September 2008. At harvest, root collar diameter, height of main shoot, number of side branches, lengths of side branches, number of leaves, fresh mass of leaves, stems, fine and coarse roots were determined. Leaf mass was determined for 5 fully expanded leaves collected at the top of the leader shoot of each tree and their areas were measured using ImageJ (http://rsbweb.nih.gov/ij/). These data were used to convert leaf mass per tree to leaf area per tree. Aliquots of plant tissues were shock-frozen in liquid nitrogen and stored at -80°C for biochemical analysis. Aliquots of roots were used for mycorrhizal assessment. Other plant tissues were dried at 60°C to determine dry mass and the relative water content [fresh mass – dry mass)*100/fresh mass].

Endophyte colonization

Two fully expanded, healthy top leaves of 20 plants per treatment of C3 and of 18 plants per treatment of C4 were harvested (21th Aug. 2008), cut into quarters and surface-sterilized for 1 min in 96 % EtOH, 3 min in 4 % NaOCl and 30 s in 96 % EtOH. The four leaf pieces were placed upside down in a Petri dish on antibiotics containing water-agar (15 g L-1 agar with 15 mg tetracycline, 100 ampicilline, 50 mg kanamycine, and 0.1 mg streptomycine) and were incubated for 7 days at 20°C in darkness (Petrini, 1986). Subsequently hyphal outgrowth of leaf pieces was scored as absent or present on each leaf piece yielding a scale from 0 %, 25 %, 50 %, 75 % and 100 % endophyte presence, respectively, per leaf in a Petri dish.

Insect sampling

Insects were captured by using a sweep net and an exhauster or were identified directly on the trees. Aphids, leaf beetle larvae and galls were quantified visually on each tree. Counting was done four times (monthly) from May to the middle of August. We recorded three leaf beetle (*Phratora vitellinae*, *Crepidodera aurata* and *Crepidodera aurea*) and one aphid species (*Chaitophorus populi*). The identification of adult insects was done in the laboratory. Due to negligible abundances, miners, galls, Homoptera and different predators were not included in statistical analysis. Leaf beetles and their larvae and other chewing insects like Symphyta larvae and caterpillars were pooled and denominated as “sum of chewing insects” and aphids and cicada were pooled as “sum of sucking insects”.

Mycorrhizal colonization

To determine colonization with ectomycorrhizal fungi fine roots were cut into small pieces and mixed. Aliquots of the mixtures were spread under a dissecting microscope (Zeiss, Stemi 2000-C) and the presence or absence of typical ectomycorrhizal mantle structures was recorded on 100 root tips per sample. To measure colonization with arbuscular mycorrhizal fungi, root samples were placed immediately after harvest in 80 % EtOH. The samples were subsequently stained with trypane-blue in lactophenol, destained and mycorrhizae detected by the presence of hyphae, arbuscules or vesicles in root tissue whose abundance was recorded by the gridline intersection method as reported previously (Ducic et al., 2009).

Genetic analysis

To control the crossing experiment the DNA of the parental trees and their offspring was analysed using 5 nuclear encoded microsatellite markers. Total DNA was extracted from young leaves using the DNeasyPlant Minikit (Qiagen, Hilden, Germany). The amount and the quality of the DNA were analyzed by 0.8 % agarose gel electrophoresis with 1x TAE as running buffer (Sambrook et al., 1989). DNA was stained with ethidium bromide and visualized by UV illumination.

For microsatellite analysis the primers PMS14, PMS16 (Van der Schoot et al., 2000), PTR2, PTR4 (Dayanandan et al., 1998), and PTR5 (Rahman et al., 2000) were used. The PCR reactions were carried out as described above with the exception that primers were labelled with the fluorescent dyes 6-carboxyfluorescein (6-FAM) or hexachloro-fluorescein phosphoramidite (HEX). Fragments were separated on the ABI Genetic Analyser in a multiplex analysis. The microsatellite alleles were recognized using the software packages Genescan 3.7 and Genotyper 3.7 from Applied Biosystems.

Microsatellite loci were scored for the analysis of genetic parameters by using the computer program GENALEX (Peakall and Smouse, 2001). The analysis confirmed seven of initially eight full-sib families. Genetic variances within and between full-sib families were calculated with Molecular Analysis of Variance (MAMOVA, www.biosis.ac.uk/smart/unix/mamova) using 99 permutations.

Biochemical analysis

The biochemical analyses were conducted as described previously (Luo et al., 2006, Luo et al., 2008) and are therefore recorded here only briefly. Frozen leaves were ground to a fine powder in a ball mill cooled with liquid nitrogen (Retsch, Haan, Germany). Material of three plants of a full-sib family in each block was pooled. Glucose, fructose, sucrose and starch were extracted in DMSO/HCl and their concentrations were determined after enzymatic conversion at a wavelength of 340nm (Schopfer, 1989). Soluble proteins were extracted in phosphate buffer and measured spectrophotometrically at a wavelength of 562nm using the bicinchoninic acid kit (BCA assay, Uptima, Montlucon, France). Bovine serum albumin served as the standard. Soluble phenolics were extracted twice in 50% methanol and measured spectrophotometrically after incubation with Folin-Ciocalteus phenol reagent at 765nm. Catechin was used for calibration. Leaf pigments were extracted in 80% acetone and measured at wavelengths of 646nm (chlorophyll b), 663nm (chlorophyll a) and 470 nm (carotenoids). Their concentrations were calculated using the extinction coefficients determined by Lichtenthaler and Wellburn (1983).

Element analysis

Dry leaves were milled to a fine powder in a ball mill (Retsch, Haan, Germany). The powder was wet-ashed in 65 % HNO_3 at 170°C for 12h. The filtrate was used for ICP-OES analysis of P, S, K, Ca, Mg, Mn, and Fe (Spectro Analytic Instruments, Kleve, Germany) after Heinrichs et al. (1986). For analysis of nitrogen and carbon contents leaf powder was weighed into 5 x 9 mm tin cartouches (Hekatech, Wegberg, Germany) and analysed in a CHNS-O element analyzer EA1108 (Carlo Erba Instruments, Milan, Italy). Acetanilide was used as the standard.

Data analysis

Data were tested for normality with the Shapiro-Wilk's test. If required, data were log-transformed to meet the assumption of normality of residuals. For data analysis herbivore data of all sampling dates were pooled. Differences between parameter means were considered significant when the *P* value of the ANOVA *F* test was less than 0.05. Univariate or multivariate analysis of variance, principle component analysis, linear mixed effects models, simple regression and graphics were carried out using the software R 2.10.0 (R Development Core Team, 2009). The experimental design with two beds divided into four blocks each required statistical analysis with linear mixed effects models. We fitted linear mixed-effects models ("lme"-function in package "nlme", Pinheiro and Bates, 2000) using maximum likelihood with genotype, soluble phenolics and relative leaf water content plus their two-way interactions as fixed factors. To account for non-independence of different plot sizes, we used the following sequence of random effects: bed, block and genotype. To account for heteroscedasticity we inspected the residuals for constant variance and normality and used variance functions (Pinheiro and Bates, 2000). For model simplification we performed step-wise backwards model selection by using the Akaike Information Criterion (AIC) (Crawley, 2007, "stepAIC"-function within the "MASS"-package, Venables and Ripley, 2002). The minimal adequate model was the one with the lowest AIC (Burnham and Anderson, 2002). Multiple comparisons among factors having a significant effect in the minimal model were calculated using Tukey contrasts with *P*-values adjusted by single-step method ("multcomp"-package, Hsu, 1996). The figures were generated with the software Origin 7.0 (Origin Lab Corp., Northampton, USA).

RESULTS

Phenotypic and genetic differences between full-sib families of P. tremula

Bud break, a trait under strong genetic control, revealed distinct differences among *P. tremula*crossings (Fig. 1A). Bud break was completed 8 days earlier in C7 than in C6. The other full-sib families showed intermediate behaviour. The full-sib families furthermore differed significantly in growth (Fig 1B) as well as in many other morphometric parameters (for details, see supplement 1) such as the number of side shoots ($P < 0.001$), cumulative lengths of side per tree ($P = 0.048$), relative leaf water content ($P = 0.003$), relative height growth ($P = 0.002$), stem diameter ($P = 0.007$), stem height ($P < 0.001$), stem biomass ($P < 0.001$), leaf biomass ($P< 0.001$), below-ground biomass ($P = 0.033$), and whole plant fresh ($P < 0.001$) and dry mass ($P < 0.001$). Significant differences among full-sib families were also found for the concentrations of some leaf nutrients and for phytochemical traits [Ca ($P < 0.001$), N ($P = 0.004$), Mg ($P = 0.004$), Mn ($P < 0.001$), P ($P = 0.002$), K ($P = 0.009$), soluble phenolic compounds ($P = 0.005$), glucose ($P < 0.001$), fructose ($P = 0.009$)], whereas C, S, Fe, starch, chlorophyll, carotenoids, and soluble protein ($P > 0.05$) were unaffected by genetic differences between the full-sib families. Fungicide treatment had no significant influence on morphometric or phytochemical parameters in aspen (Supplement 1).

To classify full-sib aspen families according to their morpho- and chemometric characteristics, principle component analysis of growth and phytochemical parameters was conducted. Three components were extracted that contributed 39.7 % (component 1), 22 % (component 2) and 21 % (component 3) of the variability. The performance of C1, C2, and C4 was strongly influenced by shoot biomass and side shoots numbers and that of C3, C5 and C7 by relative growth and Mn concentrations (1st component, Fig. 2). Only C6 was strongly affected by component 2 that was mainly defined by bud break and soluble phenolics (Fig. 2).

To investigate relationships between genetic variance of the neutral markers and two parameter sets for tree performance, i.e. green leaf chemistry (mineral nutrients, phenolic compounds, carbohydrates, pigments and protein) and tree morphology (biomass of leaves, stem, and roots, leaf area, stem height increment, stem diameter, leaf numbers,

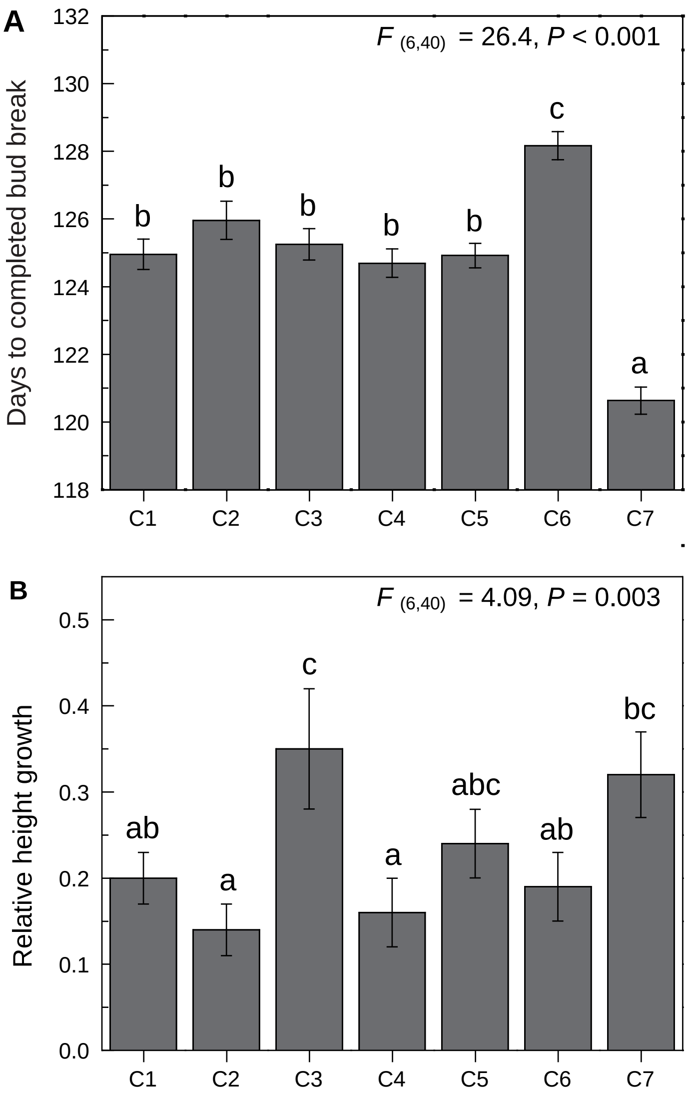

Figure 1: Bud break (**A**) and relative growth rate (**B**) of seven full sib families of aspen (*P. tremula*). (**A**) Bud break was measured as Julian days to the first fully expanded leaf on the leader shoot. (**B**) Relative growth rate was determined as annual increment in shoot height/shoot height before bud break. Data indicate means (N = 24 to 28, ± SE). Different lower-case letters indicate significant pairwise differences between respective means at $P \leq 0.05$.

whole-plant leaf area, relative leaf water content, number of side shoots, cumulative length of side shoots), Mantel tests were conducted (Tab. 1). However, neither leaf chemistry nor tree morphology showed significant relations with the genetic variance of the five neutral markers applied here. Furthermore, the neutral markers showed significantly higher molecular variance within a full-sib family than between families (Among families: $DF = 6$,

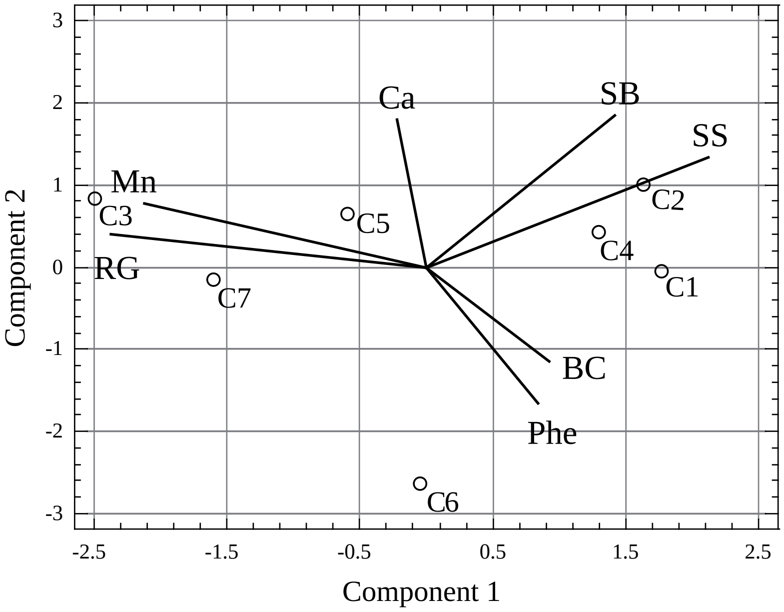

Fig. 2: Principal component analysis. The analysis was based on the following parameters: SB = stem biomass, BC = time until bud break was completed, SS = number of side shoots, RG = relative growth rate, and foliar concentrations of Phe = soluble phenolics, Mn = manganese, and Ca = calcium.

Table 1: Results of a Mantel test conducted for the relationship between genetic variance and tree performance.

Parameter group	$P_{\text{(genetic variance)}}$*
Green leaf chemistry	0.3816
Plant morphology	0.9013
All parameters	0.8957

* Genetic variances were calculated on the basis of the five neutral markers used to test the populations. Performance parameters were leaf chemistry (mineral nutrients, phenolic compounds, carbohydrates, pigments, and protein), tree morphology (biomass of leaves, stem, and roots, leaf area, stem height increment, stem diameter, leaf numbers, whole-plant leaf area, relative leaf water content, number of side shoots, cumulative length of side shoots), or all plant parameters analysed.

variation 39 %, within families: *DF* = 108, variation 69 %, PhiPT 0.3941, *P* = 0.010).

To investigate whether plant traits differed more strongly between families without common parents than between those with a common parent, the trees were combined in a matrix showing 7 combinations for common fatherhood, 2 combinations for common motherhood and 12 combinations without common parents (Supplement 2). The differences between tree traits were calculated for each combination and compared by ANOVA. Among 30 traits tested 26 % (annual stem diameter increment, number of side shoots, fructose, protein, carbon, calcium, potassium, and manganese) showed significant differences according to parenthood (Tab. 2). However, only half of them (annual stem increment, number of side shoots, fructose and protein) showed the expected stronger difference in progenies without than in those with common parents and suggests a strong paternal influence on these parameters.

Table 2: Differences in plant traits between different parenthoods.

Parameter	Differences* for			*P*
	Common father	Common mother	No common parent	
Annual stem				
Increment (mm)	-0.06 ± 0.27a	0.30 ± 0.37ab	1.02 ± 0.26b	0.042
Number of side shoots	1.32 ± 2.80a	11.17 ± 1.37b	1.71 ± 1.71b	0.004
Fructose (mg g-1 DM)	-0.09 ± 0.03a	0.25 ± 0.09b	0.20 ± 0.04ab	0.001
Protein (mg g-1 DM)	0.64 ± 0.072a	-2.87 ± 1.02b	-1.27 ± 0.57b	0.052
Carbon (%)	-0.29 ± 0.13a	0.33 ± 0.45b	0.09 ± 0.08b	0.037
Calcium (mg g-1 DM)	-2.15 ± 0.50a	-0.81 ± 0.17a	1.62 ± 0.24b	0.001
Potassium (mg g-1 DM)	-1.65 ± 0.22a	-1.12 ± 0.15ab	1.34 ± 0.48b	0.001
Manganese (mg g-1 DM)	-0.02 ± 0.00a	-0.01 ± 0.00a	0.01 ± 0.00b	0.000

* Differences were calculated for means for the combinations shown in supplement 2. Data were tested with the factors: no common parents (0), common father (1), common mother (2). Data show means (± SE). Different lower-case letters indicate significant respective pairwise differences at $P \leq 0.05$. Parameters that showed no significant differences are not shown.

Performance of full-sib families in relation to growth and defence compounds

Since growth-related parameters and soluble phenolic concentrations showed significant differences between the full-sib families, the relationship between these features was further explored. We expected that high production of phenolic compounds would consume carbon and energy, which would, thus, be unavailable for biomass production of stem and root tissues. Instead of negative trade-off, we found that the total amount of phenolics in leaves was strictly positively correlated with total plant stem + root biomass (= non-green tissue, Fig. 3, open symbols). The same was true if the relationship between the amount of phenolics and stem biomass was considered (*R* = 0.911, *P* = 0.004). We further argued that if there was a trade-off between the production of non-green tissue and phenolics in leaves, a negative relationship between the concentration of phenolics per unit of leaf tissue and the amount of non-green tissue per green tissue must be expected. However, this was not observed (Fig. 3, closed symbols). Similarly, the concentration of phenolic compounds and the relative annual growth rate were unrelated (*R* = 0.449, *P* = 0.311).

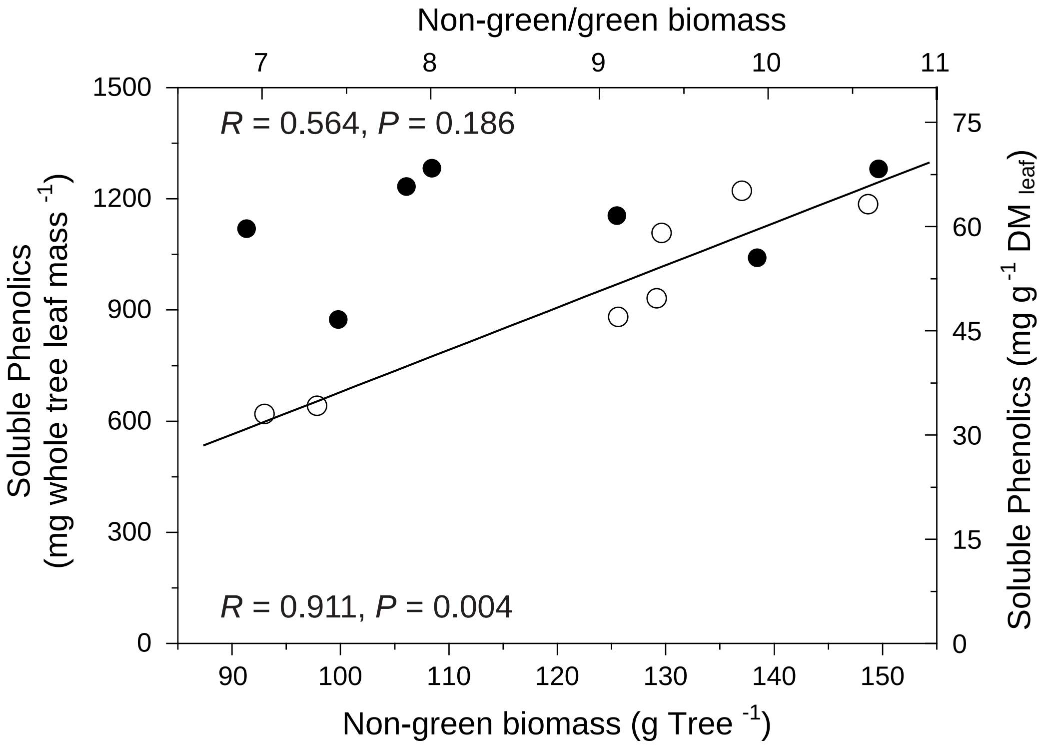

Fig. 3: Relationship between total amount of soluble phenolics in leaves and non-green (= stem + root) biomass (left and lower axis, black symbols) and between the concentration of soluble phenolics and the ratio of non-green tissue-to-green tissue (right and upper axis, white symbols).

Relationships of full-sib aspen families with interacting biota and influence of fungicide treatment

The roots of all full-sib families were colonized by arbuscular (27 %) and ectomycorrhizal fungi (16 %). Spearman correlation revealed a marginally significant negative correlation between the abundance of arbuscular and ectomycorrhizal fungi (R = -0.741, P = 0.056). Significant effects of full-sib families or of fungicide treatment on mycorrhizal abundance were not found (Supplement 1).

Endophyte colonization was only scored in two full-sib families, C3 and C4, respectively, which were characterized by a stark contrast in the concentrations of phenolic compounds (20.9 versus 30.8 mg g-1 leaf fresh mass). Between these two families no significant differences for endophyte colonization were detected (score of leaf colonization: 43 ± 8 %, P = 0.948). However, treatment with the fungicide Amistar, which was applied by soil drench, resulted in a significant decrease in endophyte colonization of leaves of both families (Tab. 3). The effect was specific for this fungal life style because mycorrhizal colonization was unaffected by the fungicide (Tab. 3, Supplement 1).

The full-sib families also differed in herbivorous insect infestation since leaf beetle larvae were significantly less abundant on leaves of the families C2 and C6 than on those of C3 (Fig. 4). There were no effects of different full sib families on aphids and the sum of sucking insect abundance (Supplement 1). A linear mixed-effect model of foliar phenolics was significant for the abundance leaf beetle larvae ($F_{(1,19)}$ = 7.22, P = 0.014) as well as for the sum of chewing insects (Fig. 5), whereas no significant effects were found for the abundance of aphids ($F_{(1,19)}$ = 0.37, P = 0.545) or the sum of sucking insects ($F_{(1,19)}$ = 0.28, P = 0.602).

Table 3: Influence of fungicide treatment on endophytes and mycorrhizas.

	Control	Fungicide *	*P*
Endophyte (% of leaf pieces)	65 ± 6	24 ± 5	< 0.001
Arbuscular mycorrhiza (% of root cells)	25 ± 3	27 ± 3	0.634
Ectomycorrhiza (% of root tips)	14 ± 1	18 ± 1	0.010

* The fungicide Amistar© was applied once a month from April to August. Colonization by endophytic fungi was scored on leaf pieces, colonization by ecto- and arbuscular mycorrhizal fungi was scored on roots. Data show means (± SE) for families C3 and C4.

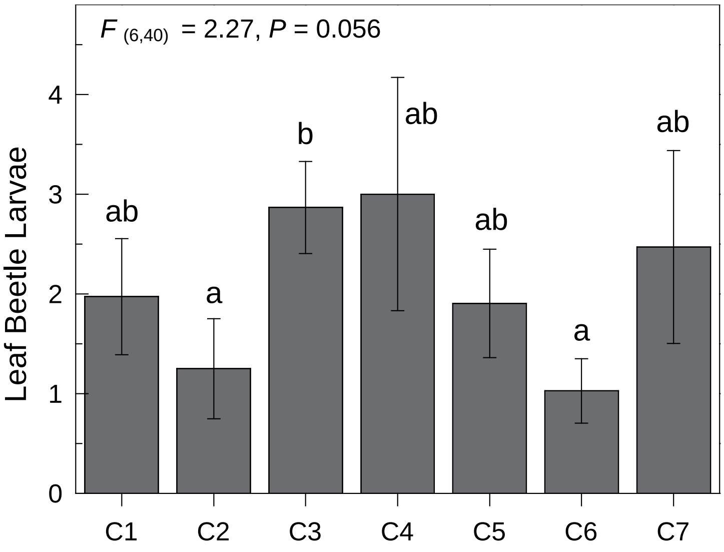

Figure 4: Abundance of chrysomelid larvae on leaves of seven full sib families of aspen (*P. tremula*). Data indicate means (N = 24 to 28, ± SE). Different lower-case letters indicate significant pairwise differences between respective means at $P \leq 0.05$.

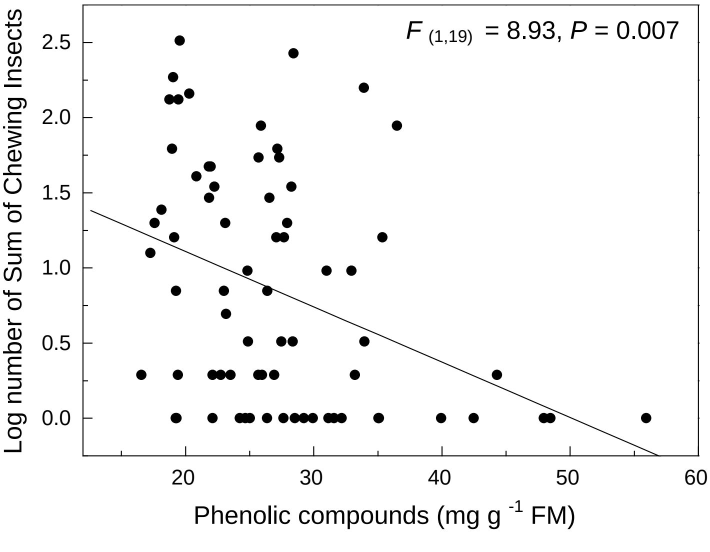

Figure 5: Relationship between the concentration of soluble phenolics in leaves and the abundance of chewing insects (note log-scale).

DISCUSSION

In this study we included a large range of morphometric and chemometric measures for phenotyping of aspen. We found significant variation in these quantitative traits in the progenies of local parental trees but correspondence between variance for neutral molecular genetic markers and phenotypic characteristics was not found. This was not unexpected since attempts to correlate genetic information based on neutral markers with traits of ecophysiological significance, e.g. timing of bud break, growth, or other quantitative traits have frequently been unsuccessful (McKay and Latta, 2002, Bekessy et al., 2003). Similarly, meta-analysis revealed only a very weak correlation between quantitative traits and molecular measures for genetic variation (mainly isozyme-based analyses, Reed and Frankham, 2001). A comparison of neutral markers, SNPs in candidate genes and quantitative phenological parameters such as bud break, bud set, seasonal increase in tree height and stem diameters in *P. tremula* genotypes from a clinal gradient also failed to detect significant correlations between molecular and phenological measures (Hall et al., 2007). In contrast to those studies, Madritch et al. (2009) found a significant correlation between genetic distance and green leaf chemistry for *P. tremuloides* clones. However, our data do not support such a relationship in *P. tremula*, probably, because the genetic variance within a family of siblings was higher than between different families. Nevertheless, some morphological and phytochemical traits showed significant parental influence. The reason for this apparent contradiction is probably that genetic variance was measured with neutral markers, whereas phenotypic characteristics are the integrative result of many functional genes.

In *P. tremula* the concentration of phenolics was under genetic control such as in *P. tremuloides* (Osier and Lindroth, 2006, Donaldson and Lindroth, 2007). Interestingly, allocation of a range of nutritional elements such as N, P, Ca, Mg and Mn was also under genetic control in *P. tremula*. N and P are major compounds in biogeochemical cycles. The elements Ca and Mg are important in ecological nutrient cycles stabilizing or counteracting decreases in soil pH (Guckland et al., 2009). Litter input of fast-degrading leaves therefore has profound effects on ecosystem processes. Whether the observed differences in leaf quality and quantities would be sufficient to influence ecosystem functions, for example, in plantations remains to be seen. With respect to Mn, genetic differences in uptake and root-to-shoot allocation have been reported for the interior and coastal race of Douglas fir (Ducic et al., 2006). The interior Douglas fir showed better performance on poor soils than the coastal provenience (Ducic et al., 2009), but in some locations its inability to limit Mn translocation to above-ground plant tissues caused severe bark diseases resulting in significant economic loss (Schöne, 1992). Our findings underline that it will be worthwhile to investigate the genetic basis of mineral nutrient allocation, especially if aspens were used for agro-forestry systems.

Leaf concentrations of phenolic compounds are important factors shaping interactions with other biota (Orians et al., 1997, Glynn et al., 2004, Whitham et al., 2006). Their concentrations vary strongly between different *Populus* species and their hybrids and these variations are inverselycorrelated with infestation with leaf galls and arthropods (Glynn et al., 2004, Whitham et al., 2006, Holeski et al., 2009). In greenhouse experiments with *P. tremuloides* phenolic glycoside concentrations were the best predictor for gypsy moth larval performance (Donaldson et al., 2006, Donaldson and Lindroth, 2007). Our data indicate that variation in leaf phenolics of *P. tremula* mediated interactions with leaf beetle larvae, belonging to the genus *Phratora* and *Crepidodera*. These chrysomelid species are generalists on salicaceous plants and not specifically adapted to utilization of phenolic glucosides like some other *Chrysomela* species (Denno et al., 1990, Glynn et al., 2004, Ikonen, 2002). High concentrations of leaf phenolic compounds provide a protection from generalist herbivores as indicated by the negative relationship between phenolics and leaf beetle larvae abundance. Our data further suggest that the costs incurred by this constitutive protection are too small to result in significant trade-off for growth or biomass production.

In this study the influence of Amistar Opti, a strobilurin-based antifungal compound, was also studied. The active agent has initially been isolated from *Strobilurus tenacelus,* a saprophytic fungus growing on pine cones (Anke et al., 1975). Although this fungicide acts against a broad number of fungal species from different classes (ascomycota, basidiomycota, oomycetes), we showed that it does not suppress mycorrhiza formation. This has also been reported for other modern fungicides (Feldman, 2003, Watson, 2006). Mycorrhizal colonization of aspens in our study was similar to that found in other field studies (Baum and Makeschin, 2000). In fact, ectomycorrhizas even tended to be more abundant in fungicide treated trees, which may be the result of reduced growth of potential competitors. Endophytic fungi, which colonize the apoplastic space of plant tissues and feed on plant carbohydrates, may be such competitors. They were strongly suppressed by Amistar Opti. Although endophytes often increase plant performance (Clay, 1996, Morse et al., 2002, Bailey et al., 2005, but see Feath and Sullivan, 2003), we did not find negative effects of their suppression on plant nutrition, growth or insect feeding in this short term study. Since trees are cultivated for several years before harvest, it will be important for future investigations to assess if reduction of endophytes has long-term negative effects.

CONCLUSIONS

We showed that full-sib aspen families exhibit significant intra-specific variation in growth and ecophysiological leaf traits and that some of these traits are clearly related to parenthood. In contrast to our expectation, production of phenolic compounds, which act as defence against generalist herbivores, did not show negative trade-off with growth. Probably, the concentrations of these compounds were too low compared with lignin production or other energy consuming processes to influence growth behaviour. Application of a systemic fungicide did neither suppress mycorrhizal colonization nor affected infestation with insects. However, a strong suppression of endophytes was found, whose long-term consequences may require attention when fungicides are used in agro-forestry plantations.

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APPENDIX

Supplement 1: Results of linear mixed-effects models for different response variables in seven full-sib aspen families (C1, C2, C3, C4, C5, C6, and C7), describing the effects of full-sib families (genotype) and of fungicide treatment. NumDF = numerator degrees of freedom, DenDF = denominator degrees of freedom. Bold font indicates significant *P*-values.

group of traits	parameter	units	genotype			
			num DF	den DF	*F*-value	*P*-value
herbivory	chrysomelidae larvae	number	6	40	2.27	0.0561
	sum of cewing insects	number	6	40	2.06	0.0800
	aphidae	number	6	40	1.40	0.2370
	sum of sucking insects	number	6	40	1.59	0.1756
mycorrhiza	EM	% colonization	6	40	0.56	0.7604
	AM	% colonization	6	25	1.41	0.2510
phytochemical leaf traits	soluble phenolic compounds	mg g-1 fresh mass	6	37	17.26	**< 0.0001**
	glucose	mg g-1 fresh mass	6	37	6.90	**0.0001**
	fructose	mg g-1 fresh mass	6	37	3.39	**0.0091**
	starch	mg g-1 fresh mass	6	37	1.03	0.4328
	chlorophyll a+b	mg g-1 dry mass	6	35	0.86	0.5323
	carotinoid	mg g-1 dry mass	6	37	2.30	0.0546
	soluble protein	mg g-1 dry mass	6	35	0.96	0.4644
leaf nutrients	Ca	mg g-1 dry mass	6	37	92.65	**< 0.0001**
	Mn	mg g-1 dry mass	6	37	7.04	**< 0.0001**
	N	%	6	37	3.83	**0.0045**
	C	%	6	37	1.00	0.2179
	P	mg g-1 dry mass	6	37	4.24	**0.0024**
	K	mg g-1 dry mass	6	37	3.39	**0.0092**
	S	mg g-1 dry mass	6	37	0.41	0.8665
	Fe	mg g-1 dry mass	6	37	2.05	0.0835
morphometric parameter	number of side shoots	number	6	40	4.87	**0.0008**
	cumulative lenghts of side shoots	cm	6	40	2.44	**0.0483**
	leaf number per tree	number	6	40	5.43	**0.0003**
	total leaf biomass	g	6	40	4.81	**0.0009**
	leaf area per tree	cm²	6	40	2.87	**0.0203**
	relative leaf water content	% of fresh mass	6	40	3.95	**0.0034**
	relative height growth		6	40	4.09	**0.0027**
	stem diameter	mm	6	40	3.53	**0.0068**
	stem height	cm	6	40	9.33	**< 0.0001**
	stem biomass	g	6	40	6.32	**0.0001**
	below ground biomass	g	6	40	2.58	**0.0333**
	plant fresh biomass (above ground)	g	6	40	5.72	**0.0002**
	whole plant dry biomass	g	6	40	5.06	**0.0006**
	days to completed bud break	days	6	40	26.40	**< 0.0001**

Supplement 1: Results of linear mixed-effects models for different response variables in seven full-sib aspen families (C1, C2, C3, C4, C5, C6, and C7), describing the effects of full-sib families (genotype) and of fungicide treatment. NumDF = numerator degrees of freedom, DenDF = denominator degrees of freedom. Bold font indicates significant *P*-values.

group of traits	parameter	units	fungicide treatment			
			num DF	DenDF	*F*-value	*P*-value
herbivory	chrysomelidae larvae	number	1	5	0.1765	0.6919
	sum of cewing insects	number	1	5	0.0937	0.7718
	aphidae	number	1	5	3.3125	0.1284
	sum of sucking insects	number	1	5	3.5309	0.1190
mycorrhiza	EM	% colonization	1	5	2.7835	0.1561
	AM	% colonization	1	5	0.9100	0.3839
phytochemical leaf traits	soluble phenolic compounds	mg g-1 fresh mass	1	5	0.0920	0.7736
	glucose	mg g-1 fresh mass	1	5	0.9350	0.2229
	fructose	mg g-1 fresh mass	1	5	0.0110	0.9207
	starch	mg g-1 fresh mass	1	5	4.0342	0.1008
	chlorophyll a+b	mg g-1 dry mass	1	5	0.0373	0.8545
	carotinoid	mg g-1 dry mass	1	5	7.2800	**0.0429**
	soluble protein	mg g-1 dry mass	1	5	0.4422	0.5355
leaf nutrients	Ca	mg g-1 dry mass	1	5	0.3508	0.5794
	Mn	mg g-1 dry mass	1	5	0.3760	0.5666
	N	%	1	5	1.8490	0.2320
	C	%	1	5	0.0000	0.8229
	P	mg g-1 dry mass	1	5	1.9620	0.2203
	K	mg g-1 dry mass	1	5	1.9140	0.2251
	S	mg g-1 dry mass	1	5	0.3880	0.5608
	Fe	mg g-1 dry mass	1	5	5.7100	0.0625
morphometric parameter	number of side shoots	number	1	5	0.3800	0.5648
	cumulative lenghts of side shoots	cm	1	5	1.1344	0.3655
	leaf number per tree	number	1	5	0.3740	0.5675
	total leaf biomass	g	1	5	2.4540	0.1780
	leaf area per tree	cm²	1	5	0.0090	0.9288
	relative leaf water content	% of fresh mass	1	5	0.9800	0.3669
	relative height growth		1	5	0.7783	0.4180
	stem diameter	mm	1	5	0.3300	0.5897
	stem height	cm	1	5	0.0600	0.8183
	stem biomass	g	1	5	1.6620	0.2537
	below ground biomass	g	1	5	0.7990	0.4123
	plant fresh biomass (above ground)	g	1	5	0.3060	0.6040
	whole plant dry biomass	g	1	5	0.5370	0.4964
	days to completed bud break	days	not tested			

(continued)

Supplement 1: Mean and standard error of different herbivorous insects as well as distinct tree traits of seven full-sib aspen families (C1, C2, C3, C4, C5, C6, and C7).

group of traits	parameter	units	C1		C2	
			mean	SE	mean	SE
herbivory	chrysomelidae larvae	number	1.97	0.60	1.25	0.51
	sum of cewing insects	number	2.22	0.59	1.36	0.51
	aphidae	number	4.13	1.00	4.94	1.70
	sum of sucking insects	number	4.25	1.02	5.11	1.71
mycorrhiza	EM	% colonization	15.96	1.86	14.42	1.68
	AM	% colonization	26.27	2.65	37.64	3.24
phytochemical leaf traits	soluble phenolic compounds	mg g-1 fresh mass	26.35	1.13	25.69	1.23
	glucose	mg g-1 fresh mass	17.79	0.39	17.67	1.28
	fructose	mg g-1 fresh mass	0.60	0.02	0.54	0.03
	starch	mg g-1 fresh mass	1.65	0.39	1.72	0.31
	chlorophyll a+b	mg g-1 dry mass	3.54	0.28	3.78	0.28
	carotinoid	mg g-1 dry mass	443.05	29.81	488.56	23.00
	soluble protein	mg g-1 dry mass	34.35	1.46	34.89	2.46
leaf nutrients	Ca	mg g-1 dry mass	12.06	0.50	16.36	0.34
	Mn	mg g-1 dry mass	49.52	1.72	48.62	2.37
	N	%	2.25	0.05	2.03	0.04
	C	%	47.22	0.19	47.00	0.15
	P	mg g-1 dry mass	1.85	0.05	1.79	0.07
	K	mg g-1 dry mass	12.42	0.51	8.23	0.42
	S	mg g-1 dry mass	1.90	0.04	1.84	0.06
	Fe	mg g-1 dry mass	85.95	2.20	84.15	4.51
morphometric parameter	number of side shoots	number	53.48	7.22	53.50	4.56
	cumulative lenghts of side shoots	cm	55.57	10.68	33.55	10.36
	leaf number per tree	number	310.90	31.48	237.96	28.91
	total leaf biomass	g	43.99	4.91	32.96	5.37
	leaf area per tree	cm²	3504.61	410.33	2795.96	641.54
	relative leaf water content	% of fresh mass	47.23	0.69	51.55	1.37
	relative height growth		0.21	0.02	0.15	0.03
	stem diameter	mm	15.29	0.44	14.66	0.48
	stem height	cm	223.29	6.72	204.04	7.01
	stem biomass	g	172.96	16.91	140.88	12.42
	below ground biomass	g	57.88	6.39	46.89	4.26
	plant fresh biomass (above ground)	g	233.40	22.31	191.73	18.28
	whole plant dry biomass	g	171.18	68.76	141.23	60.54
	days to completed bud break	days	124.95	0.45	125.96	0.57

(continued)

Supplement 1: Mean and standard error of different herbivorous insects as well as distinct tree traits of seven full-sib aspen families (C1, C2, C3, C4, C5, C6, and C7).

Group of traits	parameter	units	C3		C4	
			mean	SE	mean	SE
herbivory	chrysomelidae larvae	number	2.87	0.47	3.00	1.19
	sum of cewing insects	number	3.13	0.52	3.15	1.20
	aphidae	number	18.45	8.86	2.43	1.68
	sum of sucking insects	number	18.67	8.84	2.56	1.69
mycorrhiza	EM	% colonization	13.71	2.03	16.75	2.23
	AM	% colonization	27.75	4.24	25.72	2.91
phytochemical leaf traits	soluble phenolic compounds	mg g-1 fresh mass	20.98	0.65	30.84	1.90
	glucose	mg g-1 fresh mass	21.57	1.91	17.96	1.40
	fructose	mg g-1 fresh mass	0.68	0.03	0.66	0.02
	starch	mg g-1 fresh mass	2.17	0.61	1.82	0.47
	chlorophyll a+b	mg g-1 dry mass	3.28	0.42	3.28	0.26
	carotinoid	mg g-1 dry mass	665.64	78.55	425.39	29.91
	soluble protein	mg g-1 dry mass	32.68	1.89	32.75	2.38
leaf nutrients	Ca	mg g-1 dry mass	14.70	0.55	14.19	0.52
	Mn	mg g-1 dry mass	79.43	3.15	63.36	2.30
	N	%	2.17	0.04	2.11	0.04
	C	%	47.21	0.29	47.76	0.29
	P	mg g-1 dry mass	1.87	0.07	1.78	0.05
	K	mg g-1 dry mass	9.73	0.50	10.09	0.33
	S	mg g-1 dry mass	1.92	0.06	1.95	0.06
	Fe	mg g-1 dry mass	95.72	3.30	80.45	2.09
morphometric parameter	number of side shoots	number	43.75	3.07	55.08	3.13
	cumulative lenghts of side shoots	cm	50.78	10.90	77.59	16.25
	leaf number per tree	number	230.33	19.30	303.42	25.68
	total leaf biomass	g	32.25	3.73	45.61	5.22
	leaf area per tree	cm²	2615.82	317.86	3255.67	408.08
	relative leaf water content	% of fresh mass	47.68	0.86	47.47	0.87
	relative height growth		0.41	0.07	0.19	0.03
	stem diameter	mm	13.67	0.48	14.28	0.28
	stem height	cm	160.88	8.84	201.75	7.63
	stem biomass	g	113.41	10.93	159.25	13.30
	below ground biomass	g	38.79	2.96	54.19	3.45
	plant fresh biomass (above ground)	g	163.34	15.25	220.98	18.86
	whole plant dry biomass	g	112.11	46.30	156.69	50.71
	days to completed bud break	days	125.04	0.41	124.38	0.42

(continued)

Supplement 1: Mean and standard error of different herbivorous insects as well as distinct tree traits of seven full-sib aspen families (C1, C2, C3, C4, C5, C6, and C7).

group of traits	parameter	units	C5		C6	
			mean	SE	mean	SE
herbivory	chrysomelidae larvae	number	1.98	0.59	1.03	0.34
	sum of cewing insects	number	2.17	0.63	1.18	0.34
	aphidae	number	4.25	2.10	6.53	3.02
	sum of sucking insects	number	4.40	2.11	6.71	3.03
mycorrhiza	EM	% colonization	15.97	1.88	18.09	2.18
	AM	% colonization	31.65	4.13	15.78	1.58
phytochemical leaf traits	soluble phenolic compounds	mg g-1 fresh mass	27.61	1.65	30.76	1.59
	glucose	mg g-1 fresh mass	20.25	2.02	18.75	3.10
	fructose	mg g-1 fresh mass	0.48	0.04	0.81	0.18
	starch	mg g-1 fresh mass	1.54	0.45	1.91	0.41
	chlorophyll a+b	mg g-1 dry mass	4.06	0.23	3.21	0.18
	carotinoid	mg g-1 dry mass	503.26	25.07	418.77	23.52
	soluble protein	mg g-1 dry mass	40.02	2.43	33.49	1.85
leaf nutrients	Ca	mg g-1 dry mass	14.44	0.46	13.04	0.47
	Mn	mg g-1 dry mass	65.00	3.61	55.22	1.77
	N	%	2.12	0.03	2.08	0.04
	C	%	46.96	0.09	47.12	0.08
	P	mg g-1 dry mass	1.60	0.04	1.83	0.11
	K	mg g-1 dry mass	9.28	0.43	10.41	0.88
	S	mg g-1 dry mass	1.98	0.11	1.85	0.06
	Fe	mg g-1 dry mass	90.05	2.65	92.67	5.71
morphometric parameter	number of side shoots	number	46.04	3.35	40.96	3.60
	cumulative lenghts of side shoots	cm	63.34	15.02	21.39	4.85
	leaf number per tree	number	216.33	16.87	162.00	16.89
	total leaf biomass	g	39.58	3.86	22.60	2.73
	leaf area per tree	cm²	2435.54	275.82	1587.80	208.51
	relative leaf water content	% of fresh mass	48.72	0.89	50.56	1.01
	relative height growth		0.24	0.04	0.20	0.05
	stem diameter	mm	15.50	0.40	13.42	0.36
	stem height	cm	210.63	7.82	156.04	7.71
	stem biomass	g	144.15	9.24	91.16	8.79
	below ground biomass	g	56.52	4.13	44.51	3.17
	plant fresh biomass (above ground)	g	200.12	12.53	124.87	11.76
	whole plant dry biomass	g	146.35	45.55	103.10	37.66
	days to completed bud break	days	124.79	0.45	128.17	0.37

(continued)

Supplement 1: Mean and standard error of different herbivorous insects as well as distinct tree traits of seven full-sib aspen families (C1, C2, C3, C4, C5, C6, and C7).

group of traits	parameter	units	C7	
			mean	SE
herbivory	chrysomelidae larvae	number	2.47	0.97
	sum of cewing insects	number	2.67	1.00
	aphidae	number	3.71	1.02
	sum of sucking insects	number	3.85	1.01
mycorrhiza	EM	% colonization	17.24	2.19
	AM	% colonization	22.49	1.96
phytochemical leaf traits	soluble phenolic compounds	mg g-1 fresh mass	33.80	1.43
	glucose	mg g-1 fresh mass	28.31	2.46
	fructose	mg g-1 fresh mass	0.44	0.03
	starch	mg g-1 fresh mass	5.25	0.87
	chlorophyll a+b	mg g-1 dry mass	2.72	0.13
	carotinoid	mg g-1 dry mass	369.10	11.92
	soluble protein	mg g-1 dry mass	30.09	1.19
leaf nutrients	Ca	mg g-1 dry mass	15.70	0.39
	Mn	mg g-1 dry mass	64.83	3.10
	N	%	1.84	0.02
	C	%	47.05	0.06
	P	mg g-1 dry mass	1.54	0.02
	K	mg g-1 dry mass	7.65	0.20
	S	mg g-1 dry mass	1.64	0.03
	Fe	mg g-1 dry mass	83.55	1.49
morphometric parameter	number of side shoots	number	33.32	2.04
	cumulative lenghts of side shoots	cm	79.14	15.34
	leaf number per tree	number	231.50	15.69
	total leaf biomass	g	45.55	4.27
	leaf area per tree	cm²	2805.98	291.38
	relative leaf water content	% of fresh mass	49.26	0.77
	relative height growth		0.32	0.05
	stem diameter	mm	15.37	0.58
	stem height	cm	212.59	13.63
	stem biomass	g	139.03	13.78
	below ground biomass	g	54.23	4.08
	plant fresh biomass (above ground)	g	197.13	18.42
	whole plant dry biomass	g	147.51	51.56
	days to completed bud break	days	120.43	0.44

Supplement 2: Matrix showing combination of common fathers (1), common mothers (2) and no common parents (0) for the full-sib aspen families.

		C1	C2	C3	C4	C5	C6	C7
	parent	4x5	9x5	8x5	2x5	2x3	9x3	7x1
C1	4x5							
C2	9x5	1						
C3	8x5	1	1					
C4	2x5	1	1	1				
C5	2x3	0	0	0	2			
C6	9x3	0	2	0	0	1		
C7	7x1	0	0	0	0	0	0	

CHAPTER

3

VARIABLE RESPONSES OF LEAF BEETLE ADULTS AND LARVAE AND THEIR PREDATORS TO MORPHOLOGICAL AND GENETIC DIFFERENCES IN *POPULUS TREMULA* TREES

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ABSTRACT

1 Research on plant-herbivore interactions on willows and poplars focuses mostly on the role of leaf chemicals in Salix clones or Populus hybrids. But less is known from real-world multitrophic interactions on genetically different Populus tremula trees.

2 Here, we study seven full-sib families of aspen (P. tremula) trees, leaf beetles and natural enemies to assess the effects of plant morphological traits and herbivore-induced plant volatiles on two generations of specialized herbivores and their predators.

3 The abundances of leaf beetle adults and larvae differed between the full-sib families, the two generations in the field and trees of different morphological traits. Herbivore-induced plant volatiles changed from spring to summer, but not with aspen genetics or herbivore load. Predatory arthropods were indirectly affected by genetic aspen constitution, as their abundance closely followed the abundance of leaf beetle adults and larvae, but they were also positively and directly attracted by the volatile (Z)-3-hexenyl acetate. In spring, leaf beetles colonized only one half of the experimental field, but in the in summer, they concentrated in the second field half. This spatio-temporal shift was presumably due to (i) spring colonization from adjacent hedges and (ii) summer avoidance of tree leaves with herbivore-induced leaf responses.

4 In conclusion, our results indicate direct bottom-up effects of P. tremula trees via plant morphological and genetic traits on leaf beetles as well as indirect herbivore-mediated effects on predatory arthropods, with patterns of herbivore load changing across field site from spring to summer.

Key Words: *Populus tremula*, leaf beetle, *Phratora* sp., *Chrysomela populi*, predatory arthropods, multitrophic interactions

INTRODUCTION

Abiotic and biotic factors influence the abundance of herbivores, including weather conditions, availability of food and the impact of natural enemies (Schoonhoven et al., 2005). Plants provide resources for herbivorous insects and their natural enemies, but resource availability can change in time and space due to changing concentrations of primary or secondary metabolites and due to changing architectural properties, driven by genetic or phenotypic differences (Ehrlich and Raven, 1964, Price et al., 1980, Clark and Messina, 1998, Bernays and Chapman, 2000, Legrand and Barbosa, 2003, Schoonhoven et al., 2005, Whitham et al., 2006). The following features of host plants can influence insect distribution: the spatial and temporal distribution of host plants (Root 1973, Risch, 1981), nutritional quality and chemical defence (Coley, 1983, Coley et al., 1985, Hemming and Lindroth, 1995), plant size and architecture (Lawton and Schröder, 1977, Southwood et al., 1979, Lawton, 1983, Legrand and Barbosa, 2003), and the abundance, i.e. quantity, of the resource (Hunter, 1992).

The plant size hypothesis suggests that larger plants can be settled by more insect species and individuals than smaller host plants, because they are more likely to be found by the herbivore (Lawton, 1983). For example, Neuvonen and Niemelä (1981) showed a significant correlation between plant size and number of herbivores.

Resource abundance measured as plant biomass (number of leaves, leaf area, etc.) is suggested to represent an important factor structuring insect herbivore communities (Hunter and Wilmer, 1989, Hunter, 1992). For example, resource abundance is the main factor responsible for population oscillations of some moth and butterfly species in temperate forests (Myers and Campbell, 1976, Dempster, 1983). Plants with a higher biomass, especially of leaves and flowers, have a higher insect species richness and abundance (Teragushi et al., 1981, Marques et al., 2000).

Whitham et al. (2006) showed that the composition of arthropod communities is also correlated with the genetic structure and diversity of the host plants. The arthropod communities became more similar as their host plants were genetically more similar. Genetic differences in resistance and tolerance against herbivore damage have been found within several poplar and willow species (Denno et al., 1990, Shen and Bach, 1997). Most studies refer to differences in leaf chemistry affecting phytophagous insects, which are known to be a key factor in resistance against herbivores in salicaceous trees. For example, *P. tremuloides* shows significant genetic variation in phenolic glucosides, well known as defence compounds against generalist herbivores (Osier and Lindroth, 2006, Donaldson and Lindroth, 2007, Stevens et al., 2007). Therefore only specialist herbivores such as *Phratora vitellinae* and *Chrysomela populi* prefer to feed on salicaceous species with relatively high concentrations of phenolic glycosides in the leaves (Finet and Gregoire, 1981, 1982, Köpf et al., 1998, Ikonen, 2002). Their larvae sequester plant-derived allelochemicals, especially salicin and salicortin, to salicylaldehyde (Pasteels et al., 1983, Smiley et al., 1985, Burse et al., 2009) for effective defence against generalist predators (Hilker and Schulz, 1994, Denno et al., 1990, Palokangas and Neuvonen, 1992) but ineffective against specialist predators (Pasteels and Gregoire, 1984, Rank and Smiley, 1994, Zvereva and Rank, 2003).

Only few studies address the emission of herbivore-induced plant volatiles (HIPV) in poplar trees. Arimura et al. (2004) and Frost et al. (2007) found mono-, sesqui and homo-terpenoids, simple phenolics, and also benzene cyanides, emitted as reactions to herbivore damage. HIPVs often serve as semiochemical cues for host location of prey by predatory insects and parasitoids, and they often shape multitrophic interactions (Takabayashi and Dicke, 1996, Dicke and Baldwin, 2010).

Plant-insect interactions have mainly been studied in willows, *P. tremuloides* or in hybrids of *P. tremuloides* with *P. tremula* (Peacock et al., 1999, 2004, Arimura et al., 2004, Osier and Lindroth, 2006), whereas less information is available on its close relative *P. tremula*, especially about the influence of plant size and resource abundance on herbivorous insects.

In this study we evaluated the relationship between morphological tree traits such as tree height, leaf number and leaf toughness, HIPVs, the abundances of leaf beetle adults and larvae and of their natural enemies on genetically different poplar trees (*P. tremula*). We posed the following questions:

1) Is there any relationship between genetic differences of the poplar trees and the herbivore load and predator abundance?
2) Which morphological tree traits drive herbivore abundances? Do these traits have a direct or indirect, prey-mediated influence on natural enemies?
3) Are there differences in volatile emission between full-sib families and seasons, which can be related to the herbivore load or to predators?

MATERIAL AND METHODS

Plant materials and experimental set-up

For our experiments we used aspen (*Populus tremula*) belonging to eight full-sibling (full-sib) families C1, C2, C3, C4, C5, C6, C7, and C8 bred by a controlled crossing. The parent tree material originates from 30-year-old trees selected in Göttingen-Geismar, Central Germany (51°32´N, 9°56´E). Each full-sib family was represented by 18 trees. To characterize the genetic differences between the full-sib families, DNA of the progenies was analyzed using five nuclear encoded microsatellite markers as described in Kleemann et al. (2010). The genetic distance between the different full-sib families was calculated according to Nei (Nei, 1978) (Fig.1, Tab. 1).

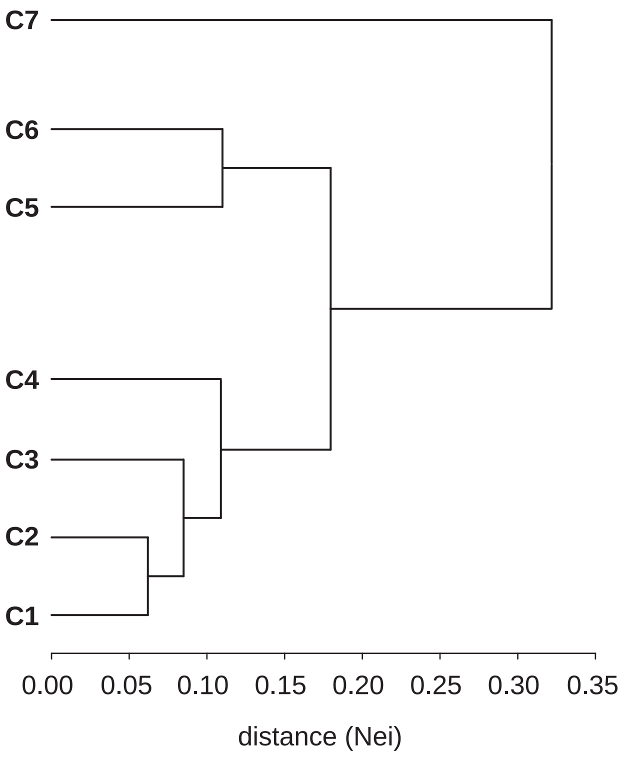

Figure 1: Genetic distance tree (according to Nei, 1978) of the seven full-sib families based on five neutral molecular markers (Kleemann et al., 2010). Hierarchical cluster analysis, average linkage.

In spring 2008 the trees were planted into 65-litre pots (Fruhstorfer soil, type T 25, Germany) with a distance of 2 m between two pots following a fully randomized design with 144 trees. The trees were watered when necessary. At the beginning of the season (2008 and 2009) the trees were fertilized (Hakophos® blau, Compo). The trees of full-sib family C8 had to be excluded from the statistical analysis as the genetic analysis revealed they cannot assign to an own full-sib family.

Table 1: Matrix of genetic distances (% according to Nei, 1978) between the seven aspen full-sib families based on five neutral molecular markers analysis.

Full-sib families	C1	C2	C3	C4	C5	C6	C7
C1	0.0						
C2	6.2	0.0					
C3	6.5	10.5	0.0				
C4	8.6	12.2	11.9	0.0			
C5	11.8	26.5	27.0	14.0	0.0		
C6	8.8	11.4	22.7	21.5	11.0	0.0	
C7	27.3	46.3	51.0	31.4	11.8	25.3	0.0

The experimental area (30 x 30m) was surrounded by a hedge (*Acer campestre, A. platanoides, Rosa canina, Crataegus* sp., *Prunus avium*) at one side, a greenhouse at the opposite side; both in a distance of 3 m to the edge of theexperimental area. The other two sides were bordered by grassland and a strawberry field.

Genetic analyses of the trees were conducted at the Buesgen-Institute of Forest Genetics and Forest Tree Breeding using same methods and markers as described in Kleemann et al. (2010).

Insect sampling

Leaf beetle adults and larvae were quantified visually on each tree, as were natural enemies such as lady bird beetles (Coccinellidae), soldier beetles (Cantharidae), wasps (solitary and social) and spiders. Counting was done twice a month (with two weeks distance between the sampling dates) from May to the end of August. Further insects were captured using a sweep net and an exhauster or were identified directly on the trees.

The adult insects were identified in the laboratory. Our sampling approach provided data on the insect species composition and estimates on abundances.

Due to negligible abundances, the adults of *Crepidodera*, *Phratora*, and *Chrysomela* species were pooled as “leaf beetle imagines”. Soldier beetles, lady bird beetles, predatory wasps, syrphid fly larvae and spiders were pooled and denominated as “sum of predators”.

Morphometric measurements

In spring (May and June) and summer (July and August) 2009 morphometric traits were measured on each tree. Tree diameter at the bottom (root collar), and the length of the main shoot (from the base to the terminal bud = tree height) were measured, the number of side branches of a tree was counted. On one side branch per tree the number of leaves was counted. To determine the number of leaves per tree we multiplied the number of side branches with the number of leaves per side branch. Leaf toughness was measured in summer on 10 randomly chosen leaves of each tree, using a Stable Micro Systems Analyser TA.XT 2. Feeding damage was estimated directly on the tree in the field and was measured on 15 leaves of each tree using the software Lamina (http://rsbweb.nih.gov/ij/).

Sampling of volatiles and extracts

Volatiles were sampled on a charcoal trap (CLSA-Filter, Daumazan sur Arize, France) using a modified push-pull headspace collection system (Tholl et al., 2006) on four randomly chosen trees of the seven full-sib families. About 50 leaves of one branch were enclosed in a plastic roasting bag (Melitta GmbH, Minden, Germany). Air was circulated through the trap by a miniature pump (Fürgut, Aichstetten, Germany) at a flow of 0.8 l min^{-1}. The operating time was 2 hrs. Adsorbed volatiles were eluted with 100 µl of dichloromethane/methanol (2:1). The solvents used were of analytical quality (both solvents Suprasolv quality, Merck/VWR, Darmstadt, Germany).

Chemicals

The following authentic standards were obtained with given purity from commercial sources: 6-methyl-5-hepten-2-one (96 %, CAS: 110-93-0, Fluka, Germany), (*Z*)-3-hexenyl acetate (98 %, CAS:3681-71-8, Aldrich, Germany), methyl salicylate (99 %, CAS: 119-36-8, Sigma-

Aldrich, Germany), β-caryophyllene (CAS: 87-44-5, Fluka, Germany), ocimene (mixture 70 % (*Z*)-ocimene + 25% limonene, 98 %, CAS: 13877-91-3, Fluka, Germany), α-humulene (98 % CAS: 6753-98-6, Fluka, Germany), and nerolidol (90 %, Aldrich, Germany).

GC-MS system and data analyses

Analyses of the volatile components (of the extract) were performed with GC-MS consisting of a gas chromatograph Agilent type 6890 (Palo Alto, USA) connected to a type 5973 quadrupole mass spectrometer with electron ionisation (EI, 70 eV). A HP-5MS column (Agilent, 30 m, 0.25 mm ID, and 0.25 µm film thickness, 5 % phenylmethylsiloxane) was used to validate the composition of the extracts. An aliquot of 1 µl was injected into a split/ splitless injector held at 250°C. The oven temperature program was 50°C for 1.5 min, followed by an increase of 7.50 °C/min to 200°C, remaining at 200°C for 5 min. Helium (purity 99,99 %) was used as the carrier gas (1 ml/min).

For identification of the constituents, mass spectra and GC linear retention indices (van den Dool and Kratz, 1963) were compared to those of authentic standards and those of given mass spectral databases of the Mass Spectral Search library of the NIST (National Institute of Standards and Technology 08, Gaithersburg, USA) and Whiley (9th Edition).

Laboratory feeding experiments

Feeding experiments were conducted in May 2009 with larvae of *Chrysomela populi*. Adults of *C. populi* were caught at three locations in the vicinity of Göttingen, Lower Saxony, Germany (Populneum Gieselwerder/Glashütte: 51°53´N 9°6´, Populneum Vaake: 51°47´N 9°64´O, Lutterberg: 51°37´N 9°64´O) and were reared in the laboratory for mating and laying eggs. For the feeding experiments first instar larvae were used.

The feeding experiments were conducted in a laboratory at room temperature (20-24 °C) under a natural day-night light rhythm. The *C. populi* larvae were placed each in a petri dish (Ø 9cm) provided with a moistened filter paper. Petri dishes were prepared with one leaf disc (Ø 20mm). These discs were punched from leaves deriving from ten randomized chosen trees of each full-sib family, standardizing the leaf area available for feeding. The experiments started in the morning and terminated after 48h. Larvae were weighed before placing into the petri dishes and after 48h. After finishing the experiments the leaf discs were scanned (300 dpi) and the area fed by the larvae was analyzed with Adobe Photoshop CS5.

Data analysis

Herbivore data of all sampling dates of May and June (spring) and of all sampling dates of July and August (summer) were pooled. All statistical analyses were carried out using the software R 2.11.1 (R Development Core Team, 2010).

To test the genotype effect on the different response variables (morphometric traits, VOCs, and herbivore abundances) we fitted generalized linear models (“glm and glm.nb”-function in package “stats and MASS”; Venables and Ripley, 2002) using quasipoisson (morphometric and VOCs variables) and negative binomial distribution (herbivore abundances) with genotype as fixed factor. We decided to compute the genetic variance with genotype as fixed factor because of the small number of neutral markers applied. Generalized linear models with negative binomial distribution were used to test effects of morphometric variables on herbivore abundances plus their two-way interaction. The same model was used to test the effects of herbivore and morphometric variables on predator abundances plus their two-way interaction. We did not include the number of leaves and tree height in one model, because of the colinearity of the two variables.

For model simplification we performed stepwise backwards model selection by using the Akaike Information Criterion (AIC) (Crawley, 2007; “stepAIC“-function within the “MASS“-package, Venables and Ripley, 2002). The minimal adequate model was the one with the lowest AIC (Burnham and Anderson, 2002). Laboratory feeding experiments and analyses of feeding damage scans were analyzed using ANOVA. Multiple comparisons among factors having a significant effect in the minimal model were calculated using Tukey contrasts with *P*-values adjusted by single-step method (“multcomp“-package, Hsu, 1996).

To investigate relationships between genetic variance of the neutral markers and the different morphometric and insect parameter, Mantel tests were conducted.

RESULTS

Morphological tree parameter and volatile organic compounds

The full-sib families differed significantly in number of leaves (spring: $F_{(6,\ 101)} = 3.3684$, $P = 0.005$, summer: $F_{(6,\ 101)} = 5.9667$, $P < 0.0001$), number of side shoots (spring: $F_{(6,\ 101)} = 2.3751$, $P = 0.03$, summer: $F_{(6,\ 101)} = 4.7211$, $P = 0.0003$), tree height (spring: $F_{(6,\ 101)} = 11.435$, $P < 0.0001$, summer: $F_{(6,\ 101)} = 12.892$, $P < 0.0001$), and in root collar diameter (spring: $F_{(6,\ 101)} = 3.1102$, $P = 0.008$, summer: $F_{(6,\ 101)} = 3.9929$, $P = 0.001$).We found no differences between the full-sib families in the relative growth rate and leaf toughness (only measured in August 2009).

One of the analyzed HIPVs methyl salicylate differ between full-sib families, but only in summer ($F_{(6,\ 21)} = 2.6613$, $P = 0.04$). All other analyzed HIPVs ocimene, 6-methyl-5-hepten-2-one, (*Z*)-3-hexenyl acetate, β-caryophyllene, α-humulene, and nerolidol did not differ significantly between full-sib families at the two sampling dates. In summer we measured significantly less amounts of these HIPVs than in spring (Appendix Tab. A1).

Results of the Mantel test showed neither significant relations between the five neutral markers and leaf beetles and their larvae, nor relations to predatory arthropods. The same is true for tree morphological and chemical (HIPV) parameters (Tab. 2).

Table 2: Results of the Mantel test conducted for the relationship between genetic variance and herbivore, predator, morphometric, and chemical (HIPV) parameters.

Parameter group	*P*-value (genetic variance)*		
	total	spring	summer
Leaf beetles and their larvae	0.579	0.111	0.862
Predeatory arthropods	0.166	0.055	0.316
Plant morphology	0.804	0.833	0.779
Herbivore-induced plant volatiles	0.742	0.822	0.403

* Genetic variances were calculated on the basis of the five neutral markers used to test the populations. Morphometric parameters of tree morphology were: tree height, stem diameter, number of leaves, and number of side shoots.

Arthopod community structure

We recorded leaf beetles of the genera *Chrysomela* (*C. populi, C. vigintipunctata*), *Crepidodera* (*C. aurata, C. aurea*), and *Phratora* (*P. vitellinae, P. laticollis*), two lady bird beetle species (*Harmonia axyridis, Coccinella septempunctata*), three soldier beetle species (*Cantharis fusca*, *Cantharis pellucida*, *Rhagonycha fulva*), and predatory wasps such as *Polistes* sp. and *Symmorphus* sp.

Covering two leaf beetle generations we counted 939 leaf beetle imagines, 3098 eggs, 5206 larvae, and 538 predatory arthropods (adults and larvae) across all seven full-sib families. *Phratora* sp. reached the highest densities of leaf beetle adults (89 %), followed by *Crepidodera* sp. (10 %), and *Chrysomela populi* and *C. vigintipunctata* (1 %). Spiders (44 %) and lady bird beetles (40 %) reached the highest densities of predatory arthropods, followed by soldier beetles (11 %). The remaining predatory arthropods were solitary and social wasps and syrphid fly larvae (together 5 %).

There were significant differences between the abundances of the spring and summer populations. Leaf beetle larvae (Δ Deviance$_{(1,\,214)}$ = 5.5866, $P = 0.018$) and the imagines of *Crepidodera* sp. (Δ Deviance$_{(1,\,214)}$ = 5.2497, $P = 0.022$) were more abundant in spring, whereas the *Phratora* sp. imagines (Δ Deviance$_{(1,\,214)}$ = 19.036, $P < 0.0001$) were more abundant in summer. Soldier beetles were more abundant in spring than in summer (Δ Deviance$_{(1,\,214)}$ = 5.2033, $P = 0.023$), but spider abundance (Δ Deviance$_{(1,\,214)}$ = 57.404, $P < 0.0001$) was higher in summer than in spring. For lady bird beetle we found no temporal pattern (Appendix Tab. A2).

Performance of the first generation (spring 2009)

Leaf beetle larvae were least abundant on full-sib family C1, different to all other full-sib families. Full-sib family C7 suffered from the highest infestation with chewing insects (Tab. 3). Abundances of leaf beetle imagines and predatory arthropods did not differ, neither between the full-sib families nor the seasons (Tab. 3).

Feeding damage of leaves was related to the abundance of leaf beetle adults ($F_{(1,\,104)} = 77.442$, $P < 0.0001$) and larvae ($F_{(1,\,104)} = 120.91$, $P < 0.0001$). Leaves of trees of full-sib family C7 were more damaged than leaves of C1 trees ($F_{(1,\,99)} = 2.5919$, $P = 0.022$), the others being intermediate without significant differences.

Table 3: Densities of leaf beetles, their larvae and predatory arthropods (arithmetic means ± standard error) on trees of different full-sib families. Results of generalized linear models (GLM) (negative binomial distributed) for leaf beetles, their larvae and predatory arthropods describing different infestations between full-sip families. Small italic letters indicate significant differences between respective means at $P \leq 0.05$. (Multiple comparisons among factors were calculated using Tukey test). Bold font indicates significant *P*-values.

Full-sib family		C1				C2				C3			
		mean	±	SE		mean	±	SE		mean	±	SE	
Leaf beetle larvae	spring	0.5	±	0.40	*(c)*	7.3	±	3.60	*(ab)*	6.4	±	2.00	*(ab)*
	summer	1.3	±	0.41	*(a)*	1.8	±	1.00	*(a)*	5.8	±	3.24	*(ab)*
Leaf beetle imagines	spring	0.6	±	0.16		1.2	±	0.30		1.1	±	0.24	
	summer	0.8	±	0.32		2.0	±	0.79		1.3	±	0.39	
Phratora sp.	spring	0.7	±	0.15		0.8	±	0.28		0.6	±	0.16	
	summer	0.6	±	0.28	*(b)*	2.0	±	0.78	*(ab)*	1.2	±	0.39	*(ab)*
Crepidodera sp.	spring	0.2	±	0.08		0.1	±	0.04		0.2	±	0.16	
	summer	0.2	±	0.12		0.0	±	0.03		0.1	±	0.05	
Predatory arthropods	spring	0.6	±	0.12		0.6	±	0.20		0.4	±	0.07	
	summer	1.0	±	0.22		0.8	±	0.16		0.9	±	0.16	
Coccinellidae	spring	0.2	±	0.12		0.3	±	0.15		0.2	±	0.06	
	summer	0.4	±	0.10		0.2	±	0.08		0.2	±	0.08	
Cantharidae	spring	0.2	±	0.06		0.1	±	0.05		0.2	±	0.06	
	summer	0.2	±	0.10		0.0	±	0.02		0.1	±	0.04	
Spiders	spring	0.1	±	0.07		0.1	±	0.05		0.1	±	0.03	
	summer	0.5	±	0.11		0.6	±	0.11		0.6	±	0.12	

(continued)

Table 3

Full-sib family		C4				C5				C6			
		mean	±	SE		mean	±	SE		mean	±	SE	
Leaf beetle larvae	spring	9.9	±	6.82	*(ab)*	3.8	±	1.04	*(ac)*	15.0	±	9.52	*(ab)*
	summer	15.3	±	9.69	*(b)*	3.0	±	1.00	*(a)*	6.7	±	2.05	*(ab)*
Leaf beetle imagines	spring	1.5	±	0.39		1.0	±	0.23		1.5	±	0.36	
	summer	3.8	±	1.77		2.2	±	0.68		1.4	±	0.45	
Phratora sp.	spring	0.8	±	0.38		0.7	±	0.18		0.8	±	0.33	
		3.7	±	1.72	*(a)*	2.2	±	0.67	*(ab)*	1.3	±	0.42	*(ab)*
Crepidodera sp.	spring	0.1	±	0.05		0.4	±	0.12		0.1	±	0.05	
	summer	0.1	±	0.06		0.0	±	0.02		0.0	±	0.03	
Predatory arthropods	spring	0.7	±	0.18		0.5	±	0.12		0.6	±	0.15	
	summer	1.5	±	0.35		1.2	±	0.24		1.0	±	0.15	
Coccinellidae	spring	0.5	±	0.16		0.3	±	0.08		0.3	±	0.16	
	summer	0.6	±	0.22		0.4	±	0.13		0.2	±	0.06	
Cantharidae	spring	0.1	±	0.06		0.1	±	0.03		0.2	±	0.06	
	summer	0.0	±	0.02		0.0	±	0.02		0.0	±	0.02	
Spiders	spring	0.0	±	0.02		0.0	±	0.02		0.1	±	0.03	
	summer	0.9	±	0.26		0.8	±	0.20		0.8	±	0.14	

(continued)

Table 3

Full-sib family		C7				results of GLM		
		mean	±	SE		Df	Δ Deviance	*P*-value
Leaf beetle larvae	spring	36.4	±	15.13	*(b)*	6	23.292	**0.0007**
	summer	3.0	±	1.51	*(ab)*	6	22.588	**0.0009**
Leaf beetle imagines	spring	1.7	±	0.49		6	7.6128	0.2679
	summer	1.6	±	0.73		6	12.288	0.0558
Phratora sp.	spring	1.5	±	0.50		6	7.4512	0.2811
	summer	1.5	±	0.70	*(ab)*	6	13.917	**0.0306**
Crepidodera sp.	spring	0.4	±	0.12		6	5.9627	0.4274
	summer	0.1	±	0.08		6	3.0618	0.8010
Predatory arthropods	spring	1.1	±	0.16		6	5.5571	0.4746
	summer	0.6	±	0.16		6	6.9808	0.3226
Coccinellidae	spring	0.8	±	0.17		6	8.0048	0.2378
	summer	0.3	±	0.07		6	5.7277	0.4544
Cantharidae	spring	0.1	±	0.04		6	2.6364	0.8529
	summer	0.1	±	0.04		6	3.1308	0.7923
Spiders	spring	0.1	±	0.06		6	2.4364	0.8755
	summer	0.3	±	0.09		6	6.1180	0.4101

Performance of the second generation (summer 2009)

On trees of the full-sib family C4 we counted significantly more leaf beetle larvae (of all genera) than on full-sib family C1, C2 and C5, but for leaf beetle imagines (of all genera) we detected no differences between the full-sib families. The genus *Phratora* showed a higher infestation of the trees of full-sib family C4 than C1 (Tab. 3). There were also no significant differences between the full-sib families regarding all predatory arthropods.

Feeding damage of leaves was less in summer than in spring, but significantly related to the abundance of leaf beetle imagines ($F_{(1, 105)} = 6.8282$, $P = 0.01$) and larvae ($F_{(1, 105)} = 4.1781$, $P = 0.04$). Leaves of trees of full-sib family C1 were less damaged than leaves of C4, C6 and C7 trees ($F_{(1, 100)} = 3.9327$, $P = 0.001$).

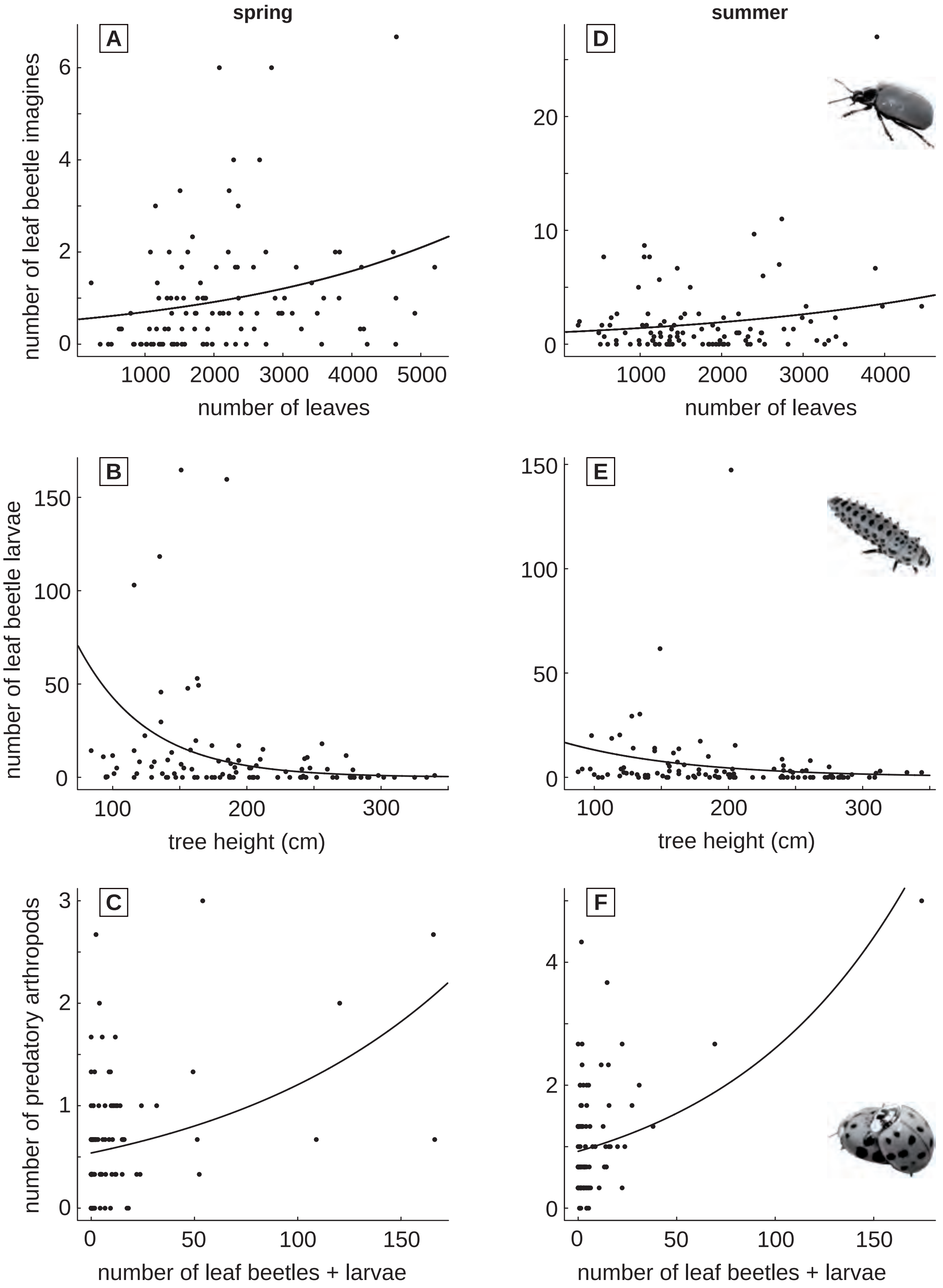

Figure 2: Relationship between morphometric parameters of polar trees and herbivore abundance (**A**, **B**, **E**, **D**), herbivore and predator abundance (**C**, **F**) in spring (**A-C**) and summer (**D-F**). **A** and **D**: Relationship between leaf beetle imagines and number of leaves. **B** and **E**: Leaf beetle larvae in relation to tree height (Generalized Linear Models – negative binomial distributed). **C** and **F**: Number of leaf beetle imagines and larvae in relation to the abundance of predatory arthropods (Generalized Linear Models - quasipoisson distributed).

Tree features and arthropod community

Both generations of leaf beetle imagines were significantly positively related to the total number of leaves (spring: Δ Deviance$_{(1,\ 106)}$ = 8.7281, P = 0.003, summer: Δ Deviance$_{(1,\ 106)}$ = 8.335, P = 0.004) (Fig. 2A, D), while tree height had a negative influence on the first, but not the second generation of leaf beetle imagines (Δ Deviance$_{(1,\ 106)}$ = 4.58, P = 0.03). Leaf beetle larvae were also negatively related to tree height (spring: Δ Deviance$_{(1,\ 106)}$ = 24.658, P < 0.0001, summer: Δ Deviance$_{(1,\ 106)}$ = 14.352, P = 0.0002) (Fig 2B, E), but not to the number of leaves per tree. Adults and larvae were negatively affected by leaf toughness (adults: Δ Deviance$_{(1,\ 106)}$ = 6.394, P = 0.01, larvae: Δ Deviance$_{(1,\ 106)}$ = 13.574, P = 0.0002).

In spring predatory arthropods were positively influenced by the number of leaf beetle imagines ($F_{(1,\ 106)}$ = 8.1996, P = 0.005) and leaf beetle larvae ($F_{(1,\ 106)}$ = 13.77, P = 0.0003) (Fig. 2C), as well as (*Z*)-3-hexenyl acetate (HIPV) (Δ Deviance$_{(1,\ 26)}$ = 5.8072, P = 0.016) (but neither by number of leaf beetle eggs, nor by one of the morphological parameters of the trees.

In summer we found the positive relationship between predatory arthropods and leaf beetle adults ($F_{(1,\ 106)}$ = 18.945, P < 0.0001) and their larvae ($F_{(1,\ 106)}$ = 18.833, P < 0.0001) (Fig. 2F) again, and also with leaf beetle eggs ($F_{(1,\ 106)}$ = 28.366, P < 0.0001). The factor genotype of full-sib families did not affect relationships between herbivores and morphological parameters or between predators and herbivores.

Spatial distribution across the field site

In spring leaf beetle larvae were more abundant at the field side adjacent to the hedge (“HA”) than at the field side adjacent to the greenhouse (“GA”) (“HA” 18.6 ± 5.1, “GA” 3.0 ± 0.8). In the second generation in summer we detected a higher abundance of leaf beetle larvae at the field side adjacent to the greenhouse (“HA” 1.7 ± 0.4, “GA” 9.0 ± 3.0). We found the same spatial distribution pattern for leaf beetle adults (spring: “HA” 1.3 ± 0.2, “GA” 0.7 ± 0.1, summer: “HA” 0.8 ± 0.1, “GA” 2.9 ± 0.6) and predatory arthropods (spring: “HA” 0.7 ± 0.1, “GA” 0.5 ± 0.1, summer: “HA” 0.8 ± 0.1, “GA” 1.3 ± 0.1) (Fig. 3).

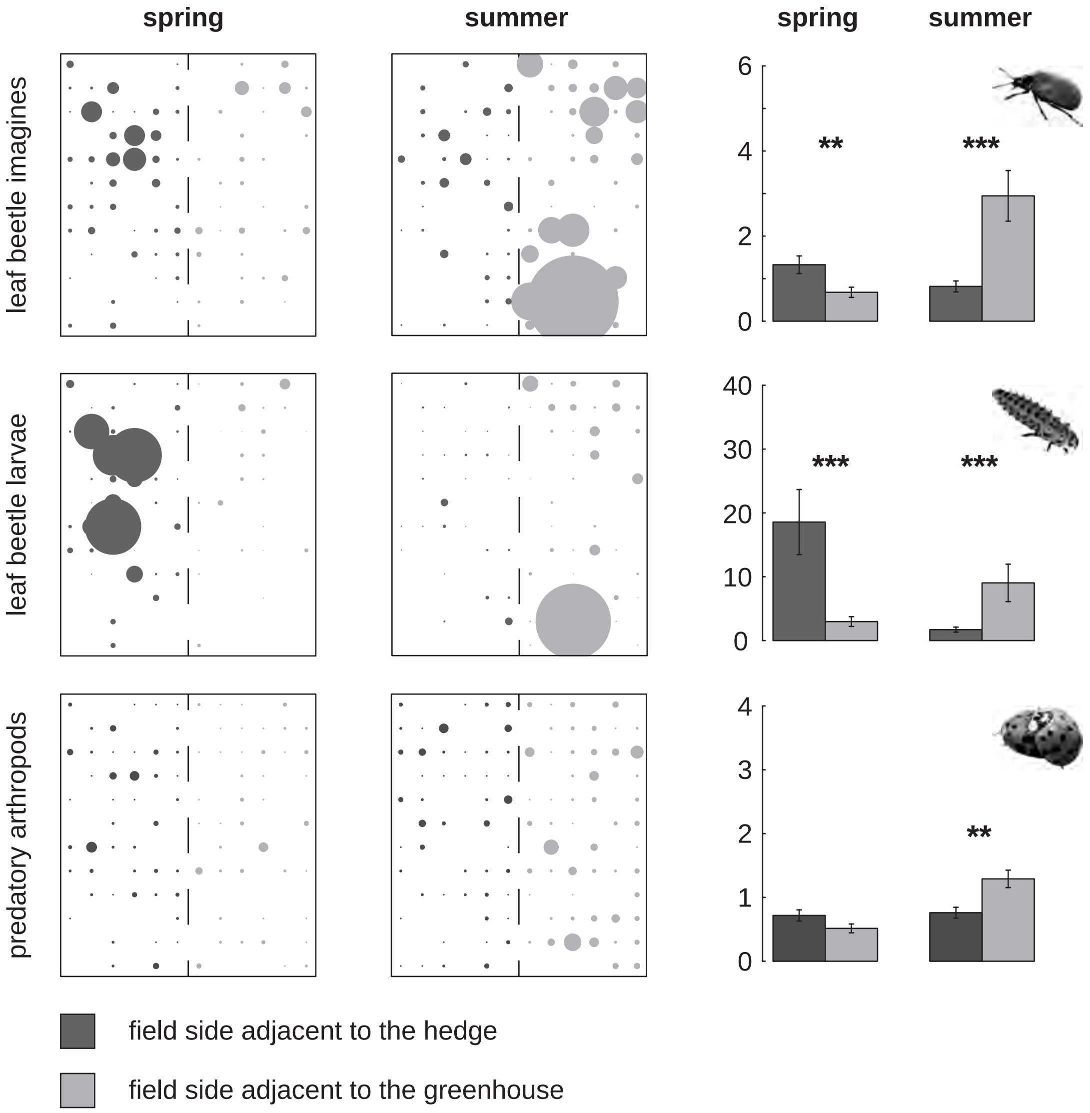

Figure 3: Spatial distribution of leaf beetle larvae, leaf beetle imagines, and total number of predatory arthropods on the experimental field in spring (May and June) and summer (July and August) 2009. As bigger a circle as more specimens were found on a tree. Bargraphs show the result of statistical analysis of distribution pattern. ** $P \leq 0.01$, *** $P \leq 0.001$. (Generalized Linear Models).

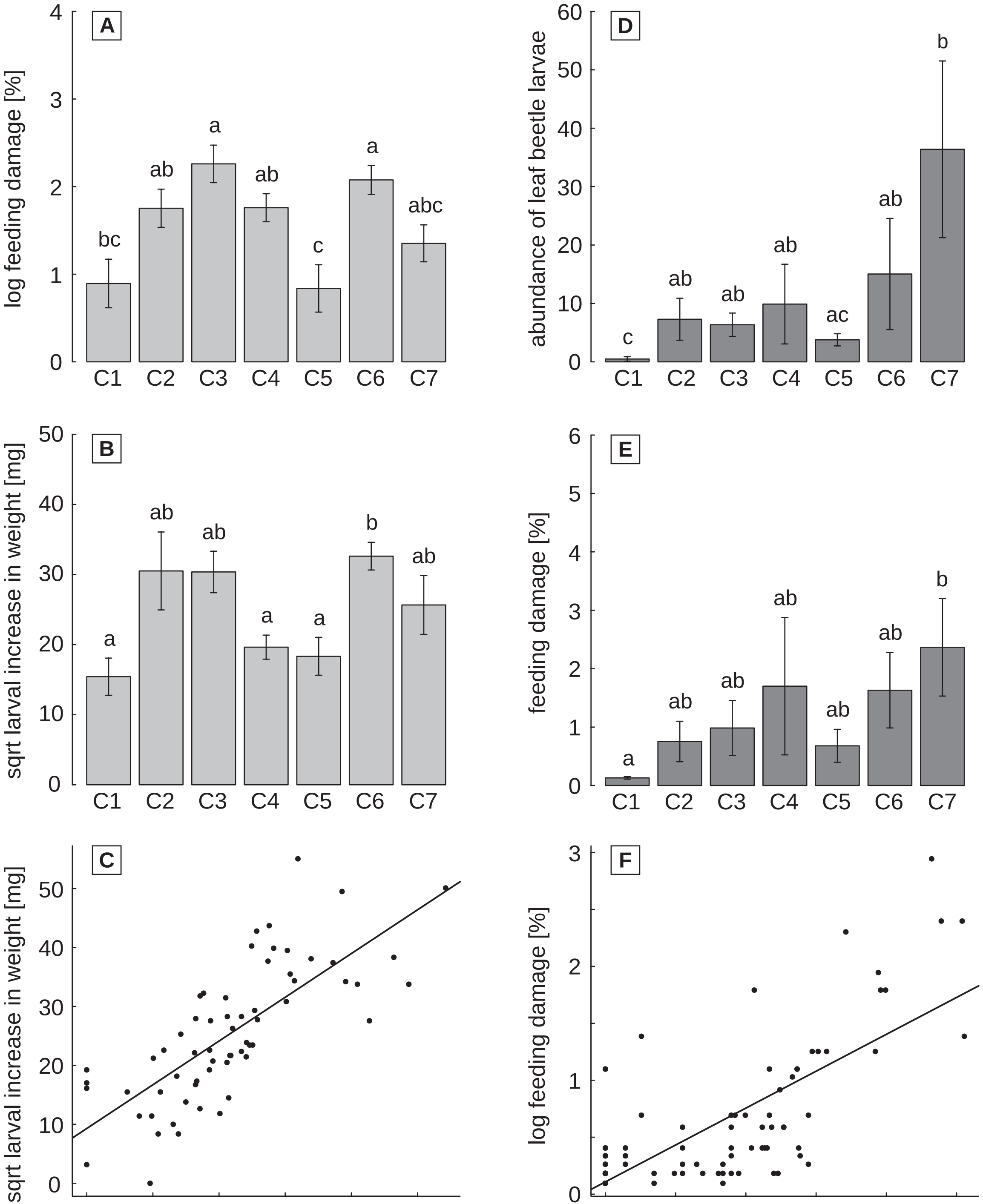

Figure 4: **A**-**C**: Laboratory feeding tests with *Chrysomela populi* larvae. **A**: Percentage of larval feeding damage. **B**: Increase in weight of *C. populi* larvae after 48h. **C**: Correlation between feeding damage and larval increase in weight. **D**-**F**: Survey of larvae abundances and distribution on seven full-sib families in the field in spring 2009. **D**: Abundance of leaf beetle larvae on trees of seven full-sib families of aspen (*P. tremula*). **E**: Feeding damage of leaves on the experimental field. **F**: Correlation between the number of leaf beetle larvae and feeding damage. Different letters indicate significant differences between respective means at $P \leq 0.05$. C1-C7 = different aspen full-sib families. (Laboratory experiments: ANOVA; Field study: Generalized Linear Models. Multiple comparisons among factors were calculated using Tukey test).

Insect feeding – laboratory vs. field results

Laboratory feeding experiments were conducted in parallel to field samples in May. Herbivory data from the field came from four records between May and June 2009.

In the laboratory experiment the *Chrysomela populi* larvae fed more leaf area from C3 and C6 than C1 and C5. Herbivory damage level in C2 and C4 was only different to C5. The larval increase in weight was related to the feeding area of the leaf discs (Fig. 4C).

In the field, trees of full-sib family C7 were significantly more damaged than of C1 (Fig. 4E). The feeding damage in the field increased significantly with the number of leaf beetle larvae (Fig. 4F).

DISCUSSION

In this study we investigated the influence of genetic variation of seven *Populus tremula* full-sib families on leaf beetle adults and larvae as well as on predatory arthropods. We tested whether morphological and genetic traits can be used to predict abundances of herbivores and predators. Despite genetic distances of 6 to 51 % we found no relationship between molecular markers and morphological tree parameters or the abundances of herbivores and predators. This is probably due to the fact that genetic variance was measured with neutral markers, whereas phenotypic characteristics are the integrative result of many functional genes. This result supports findings that genetic information based on neutral markers is often little related to traits of ecophysiological significance, such as timing of bud break and growth (McKay and Latta, 2002, Bekessy et al., 2003). In contrast, Madritch et al. (2009) found a correlation between genetic distance and green leaf chemistry for *P. tremuloides* clones.

The leaf beetle species in this study are all adapted to the chemical defence of poplar trees (the genera *Phratora* with *P. vitellinae,* and *P. laticollis*, *Crepidodera* with *C. aurea* and *C. aurata* and *Chrysomela* with *C. populi* and *C. vigintipunctata*) and can be mostly found on salicaceous species with high concentrations of leaf phenolic compounds (Gregoire, 1978, Finet and Gregoire, 1981, 1982, Orians et al., 1997, Köpf et al., 1998, Ikonen, 2002).

Kleemann et al. (2010) showed for *P. tremula* trees of the seven full-sib families, which were examined also in this study that variation in leaf phenolics mediated interactions with leaf beetle larvae of the genus *Phratora* and *Crepidodera*. Generally, high concentrations of leaf phenolic compounds provide protection against generalist herbivores and are important factors shaping trophic interactions and insect community structure on salicaceous trees (Orians et al., 1997, Glynn et al., 2004, Whitham et al., 2006). This might be the reason for the differences of feeding preferences of *Chrysomela populi* larvae in our laboratory feeding experiments, which showed a different pattern to the leaf beetle larvae abundance on the trees in the field.

The dependency of leaf beetles on resource quantity (number of leaves) and quality (leaf toughness) suggests bottom-up effects. This is in line with the resource quantity hypothesis of Hunter (1992), which suggests that plants with a higher biomass have a higher insect species abundance (Marques et al., 2000). The negative influence of leaf toughness on leaf beetles supports results showing a negative influence of leaf toughness on *Plagiodera versicolora*, a leaf beetle species on willows (Raupp, 1985).

Leaf beetle larvae of both generations and imagines of the second generation were negatively affected by tree height. This matches with observations by Kelly and Curry (1991) that larvae of *P. vulgatissima* tend to be located on leaves near the bottom and to be more abundant on internal than on distal branches. But the results are in contrast to the plant size hypothesis by Lawton (1983). For example Neuvonen and Niemelä (1981) showed a significant correlation between plant size and number of herbivores. In the case of leaf beetle larvae a possible explanation may be that leaf beetles prefer lower places on a tree, possibly a strategy of enemy avoidance (e.g. birds).

The majority of the detected predatory arthropods are known as generalists, although the salicin-using leaf beetle larvae are defended by salicylaldehyde, which is a strong deterrent against generalist predators (Denno et al., 1990, Smiley, 1991, Palokangas and Neuvonen, 1992). Our results suggest indirect effects of plants on predatory arthropods mediated through the abundance of herbivores. Similarly, Kagata et al. (2005) describe indirect bottom-up effects of leaf beetle larvae on a ladybird beetle when contrasting cut with uncut willow stands. The regeneration of cut willows shortened the developmental time of the leaf beetle and thereby also of the ladybird beetle. In addition to such bottom-up effects, top-down effects through predation pressure from predatory arthropods appear to be likely.

A chemical mechanism of plants shaping multitrophic interactions is the emission of herbivore-induced plant volatiles (HIPVs) after an infestation with herbivores. HIPVs often serve as semiochemical cues for host location of prey as is known from many predatory insects and parasitoids (Takabayashi and Dicke, 1996, Dicke and Baldwin, 2010). Our results showed a significant relationship of (*Z*)-3-hexenyl acetate to predators. (*Z*)-3-hexenyl acetate is known to be released after insect damage (Dicke et al., 1990, Rose et al., 1996) and has been found to be an attractant to predators (James, 2005) and parasitoids (Gouinguené et al., 2005). However, we did not find differences between the analyzed HIPVs of the seven full-sib families. Further, we did not find a relationship between the abundance of herbivorous insects and the emission of volatiles, which is in contrast to other studies. Arimura et al. (2004) and Frost et al. (2007) describe the emission of different mono-, sesqui- and homo-terpenoids, simple phenolics, and benzene cyanides in poplar hybrids as a reaction to herbivore damage.

We found a spatio-temporal shift of the abundances of leaf beetles on poplar trees on the experimental field. Peacock et al. (1999) described differences in spatio-temporal distribution of *Phratora vulgatissima* driven by the plantation design. In our study differences between spring and summer may be due to induced changes of leaf quality of the trees that had a high infestation in spring (which was independent of the genetic origin of the trees). Lindroth and Kinney (1998) reported short term induction of phenolic compounds in *P. tremuloides* in response to feeding damage by *Lymantria dispar*. A study by Stevens and Lindroth (2005) proved that *P. tremuloides* trees accumulate increased loads of phenolic glycosides in the leaf tissue eight weeks after previous insect damage of trees. These results could explain the strong field side shift of the leaf beetles on our experimental field. In spring, leaf beetle imagines were most abundant at the field half adjacent to a hedge, whereas in summer, most leaf beetles were found on the field half adjacent to the greenhouse. Hedges serve as overwintering site for leaf beetles when the host trees are too small (Kelly and Curry, 1991, Kendall et al., 1996, Kendall & Wiltshire, 1998). This may explain spring colonization of the field half nearby hedges.

We found differences in the abundances of leaf beetle imagines and larvae between trees of seven full-sib families that were not related to the genetic variance of the molecular markers. Instead, we detected a positive relationship between resource quantity and leaf beetle adults as well as inverse relationships between tree height and leaf beetle larvae. In addition, leaf toughness (resource quality) had a strong effect on leaf beetle imagines and larvae. Our results indicate direct bottom-up effects of *P. tremula* trees via plant morphological and genetic traits on leaf beetles as well as indirect herbivore-mediated effects on predatory arthropods, with patterns changing across the field site from spring to summer.

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APPENDIX

Table A1: Results of generalized linear models (quasipoisson distributed) for quantity differences of herbivore-induced plant volatiles between full-sib families and between spring and summer (independent from the full-sib families). Bold font indicates significant *P*-values.

			Full-sib family difference			Spring-summer difference		
Compound		N	Df	*F*-value	*P*-value	Df	*F*-value	*P*-value
Ocimene	spring	28	6	1.7159	0.1666			
	summer	28	6	0.6954	0.6561	1	13.507	**0.0005**
(*Z*)-3-Hexenyl acetate	spring	28	6	0.8043	0.5778			
	summer	28	6	0.5808	0.7415	1	25.862	< **0.0001**
6-Methyl-5-hepten-2-one	spring	28	6	1.3869	0.2657			
	summer	28	6	1.0727	0.4097	1	47.954	< **0.0001**
Methyl salicylate	spring	28	6	2.1619	0.0885			
	summer	28	6	2.6613	**0.0443**	1	6.383	**0.0145**
β-Caryophyllene	spring	28	6	0.8836	0.5240			
	summer	28	6	2.0039	0.1106	1	4.824	**0.0324**
α-Humulene	spring	28	6	0.7065	0.6479			
	summer	28	6	1.5220	0.2195	1	9.842	**0.0028**
Nerolidol	spring	28	6	1.6327	0.1876			
	summer	28	6	1.0449	0.4252	1	6.399	**0.0144**

Table A2: Densities of leaf beetles, their larvae and predatory arthropods in spring and summer 2009 (arithmetic means ± standard error).

		∑	mean	±	SE
Leaf beetle eggs	spring	199	0.6	±	0.21
	summer	2899	8.9	±	1.93
Leaf beetle larvae	spring	3443	10.6	±	2.68
	summer	1763	5.4	±	1.54
Leaf beetles	spring	323	1.0	±	0.12
	summer	616	1.9	±	0.30
Phratora sp.	spring	250	0.8	±	0.12
	summer	585	1.8	±	0.29
Crepidodera sp.	spring	66	0.2	±	0.04
	summer	28	0.1	±	0.02
Predatory arthropods	spring	199	0.6	±	0.06
	summer	334	1.0	±	0.08
Coccinellidae	spring	111	0.3	±	0.05
	summer	104	0.3	±	0.05
Cantharidae	spring	45	0.1	±	0.02
	summer	15	0.0	±	0.02
Spiders	spring	24	0.1	±	0.02
	summer	212	0.7	±	0.06

CHAPTER

4

THE ALLOMONE OF LEAF BEETLE LARVAE (SALICYLALDEHYDE) ATTRACTS EXPERIENCED *HARMONIA AXYRIDIS* AND OTHER PREDATORS

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submitted to *Biological Control*

ABSTRACT

Infochemical cues emitted by herbivore prey or herbivore-infested plants are often used by specialist and generalist predatory arthropods to find and locate their prey. We studied in a field experiment whether salicylaldehyde, released as a defense compound (allomone) by leaf beetle larvae can attract generalist predators. The generalist coccinellid beetle *Harmonia axyridis* and the cantharid beetles *Cantharis pellucida* and *Rhagonycha* sp. were highly attracted by an experimentally dispenser releasing salicylaldehyde. Similarly to the beetles the scorpionflys (Panorpidae) were highly attracted to salicylaldehyde.

Laboratory olfactometer tests with experienced *H. axyridis* imagines confirmed the results of the field study, as they were also attracted by salicylaldehyde. Naïve imagines, however, were deterred by salicylaldehyde. These results indicate that the sensory capability to perceive volatiles from prey can differ according to prior experience.

In conclusion, salicylaldehyde of leaf beetle larvae is not only an allomone for generalist predators, but can also attract experienced generalist predators using salicylaldehyde as kairomone.

Key Words: generalist predators, salicylaldhyde, allomone, *Harmonia axyridis*, *Chantharis pellucida*

INTRODUCTION

Generalist predators are important for pest control in agriculturally and forestry systems and their efficiency are often shaped by semiochemicals such as pheromones and allomones released by insects, and herbivore induced plant volatiles (Dicke and Vet, 1998, Dicke and Baldwin, 2010). It is known that in trophic systems with strong chemical defense mechanisms specialist predators and parasitoids can overcome the defense mechanisms of herbivorous insects, using their semiochemicals for host finding and location. This is documented for many parasitoids (Rutledge, 1996, Quicke, 1997) and predatory true bugs (Weissbecker et al., 2000), solitary and social wasps (Hendrichs et al., 1994, Jander, 1998), predatory beetles such as clerid beetles of the genus *Thanasimus* (Erbilgin and Raffa, 2001) and aphidophagous Coccinellidae (Raymond et al., 2000). *Harmonia axyridis* imagines are known to locate their prey by means of olfactory as well as visual cues (Obata, 1986, Verheggen et al., 2007).

In our tritrophic system consisting of *Populus tremula*, salicin-sequestering Chrysomelinae and their predators only specialist predators are attracted by the beetle's larvae allomone, salicylaldehyde. Salicylaldehyde is sequestered by leaf beetle larvae feeding on willow and poplar, which are chemically defended by phenolic glucosides, especially salicin and its derivatives (Pasteels et al., 1983, Smiley et al., 1985, Köpf et al., 1998, Tremonia et al., 2001). Specialist predators, such as phorid flies (Zvereva and Rank, 2004), syrphid flies (Smiley, 1991, Rank and Smiley, 1994, Köpf et al., 1997, Gross et al., 2004), the sawfly *Tenthredo olivacea* (Pasteels and Gregoire, 1984) and a potter wasp from the genus *Symmorphus* (Smiley, 1991, Sears et al., 2001) are attracted by salicylaldehyde, which is used as a kairomone for host finding. However, salicylaldehyde acts as a repellent against several generalist predators such as ants (Wallace and Blum, 1969, Kearsley and Whitham, 1992, Hilker and Schulz, 1994), ladybird beetle imagines and larvae (Denno et al., 1990, Smiley, 1991, Cha et al., 2009), a praying mantis (Cha et al., 2009), and spiders (Palokangas and Neuvonen, 1992). Moreover, salicylaldehyde provides antimicrobial defense for leaf beetle larvae against bacteria (Gross et al., 2002, 2008).

Because of the strong chemical defense and the specialization of its plant feeders, the trophic system consisting poplars and willows (Salicaceae), salicin-sequestering leaf beetles (Chrysomelinae) and their natural enemies represents an interesting case for the chemical ecology of tritrophic interactions.

To the best of our knowledge, there are no published results on olfactory responses of generalist predators such as soldier beetles and lady bird beetles to salicylaldehyde. The aim of this study was to determine whether there are attractant or deterrent effects of salicyladehyde to generalist predatory beetles. Additional we ask whether there are differences between experienced and naïve predators.

MATERIAL AND METHODS

Field bioassays

We used funnel traps modified from those described by Ruther et al. (2000) and Tolasch et al. (2007). Two transparent plexiglass sheets (12 cm height × 14 cm width) were arranged crosswise over a 120-mm powder funnel. These sheets were used as baffle. Baffle and powder funnel were connected to a 250 ml polyethylene collection bottle. As dispensers we used 0.2 ml PCR tubes (Thermo-Tube™, Peqlab, Erlangen, Germany). Dispensers were prepared as described in Tolasch et al. (2007), and each tube was filled with 5 mg salicylaldehyde (99 %, CAS: 17754-90-4, Aldrich, Steinheim, Germany). Dispensers were mounted on a thread in a gap at the top between the plastic sheets (Fig. 1). The collecting bottles were filled with brine (100 ml) to preserve the insects and minimize the possible attraction of insects to the already captured individuals.

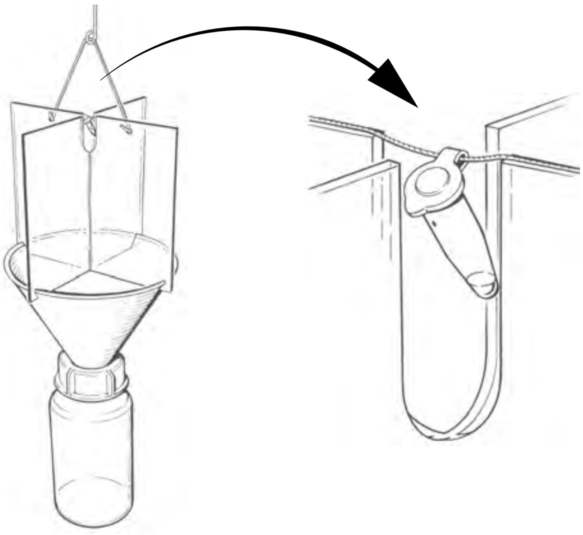

Figure 1: Funnel trap used for field experiments. Detail of the mounted salicylaldehyde dispenser. (Tolasch et al., 2007, drawing by B. Schmid, University of Hohenheim)

The field bioassays were performed from beginning of May to end of August 2009 and from end of May to end of August 2010 in an area with a young seral stage in the vicinity of Göttingen (Lower Saxony, Germany) near Lutterberg

(51°37´N 9°64´O). The study is located beneath a high-tension line, the reason why the trees were cut at regular intervals. Predominant tree species were *Populus tremula, Betula sp*. and *Salix caprea*. Trees varied in their height between one and four meter. The *P. tremula* trees had a high infestation with poplar leaf beetles (*Chrysomela populi*) and their larvae.

A total of 8 traps were grouped into 4 sets, each set containing a baited trap and a non-baited control. Traps were suspended approximately 1.5 – 2 m above the ground. The distance between traps within one set was about 5 m, whereas the distances between the sets were ca. 50 – 100 m. Traps were controlled weekly, captured beetles were removed, and the brine was replaced. Determination was done in the laboratory and collected beetles were identified to species level using Freude et al. (1967, 1979).

Laboratory bioassays

Olfactometer bioassays were conducted with *Harmonia axyridis* imagines collected in nature (experienced) and imagines emerged from pupae in the laboratory (naïve). Both (imagines and pupae) were collected on an experimental field with *Vicia faba* of the Reinshof near Göttingen (51°29´N 9°55´O). Beetles and pupae were reared in the laboratory at 20°C with a natural day and night rhythm. After emerging adults were reared at 20°C for few days and were fed with pieces of the yellow mealworm *Tenebrio molitor*.

The reaction of *H. axyridis* to salicylaldehyde stimuli was tested in a static four-chamber-olfactometer as described in Steidle and Schöller (1997) and Collatz et al. (2009). We did not discriminate between females and males, because both sexes predate on insects in the same way. The olfactometer experiments were carried out with a 10^{-3} concentration (w/w) of saliylaldehyde in order to achieve physiological meaningful concentrations. Dilutions of synthetic salicylaldehyde standards were prepared in paraffin oil (Uvasol®, spectrosc. qual., high visc., Merck, Darmstadt, Germany). Ca. 100 µl of standard dilution or paraffin oil as control were dropped on 2 cm^2 filter paper pieces (Schleicher & Schuell, Dassel, Germany).

One chamber of the olfactometer was loaded with a Petri dish containing a filter paper soaked with ca. 100 µl salicylaldehyde dilution. The opposite chamber contained a Petri dish with a filter paper soaked with ca. 100 µl paraffin oil as control. The other two chambers remained empty as transition zones. The olfactometer was illuminated from above using red light. At the beginning of each experiment, a beetle was placed in the center of the walking arena. The walking and resting positions of the beetles were recorded for 600 s by using the computer software “The Observer 2.0” (Noldus, Wageningen, The Netherlands, 1990). After each test all chambers and the walking arena were cleaned with ethanol (70 %). In order to avoid biased results due to side preferences of beetles the position of the olfactometer was rotated clockwise. Bioassays with naïve beetles were performed when beetles were 2 – 4 days old. The age of field collected beetles was unknown. Naïve beetles were freshly collected from rearing jars.

Statistical analyses

Statistical analyses were carried out using the software R, Version 2.11.1 (R Development Core Team, 2010). Data were tested for normality with the Shapiro-Wilk’s test. If required, data were log-transformed to meet the assumption of normality of residuals. With regard to the field bioassays we fitted linear mixed-effects models (“lme”-function in package “nlme”, Pinheiro and Bates, 2000) using maximum likelihood with the salicylaldehyde – control treatment as fixed factor. To account for non-independence of different sampling dates and the traps, we used the following sequence of random effects: sampling date and trap. The residuals were inspected for constant variance and normality to account for heteroscedasticity (Pinheiro and Bates, 2000).

We analyzed the duration time of beetles spent in the test and control fields in the olfactometer experiments using Wilcoxon-matched pairs test.

RESULTS

Field bioassays

In the field bioassays, a total of 34 Coccinellidae were caught with the salicylaldehyde baited traps and five beetles were caught in the non-baited control traps in the two years (Fig. 2). The most abundant species of coccinellid beetles was *Harmonia axyridis* in salicylaldehyde baited traps. A total number of 88 Cantharidae were determined in the baited traps, whereas in non-baited controls only three cantharid beetles were found. We identified *Cantharis pellucida* (50) and *Rhagonycha* sp. (36) as most abundant species. In the salicylaldehyde baited traps, Over both seasons 91 Panorpidae were caught in the salicylaldhyde baited traps and only 12 in control traps. Results separated for each of the two years are shown in Table 1. All results, combined data as well as the data of the single years, show highly significant differences between salicylaldehyde baited and non-baited control traps (Tab. 1, Fig. 2).

Laboratory bioassays

Table 1: Number of specimens of predatory beetles and scorpionflies caught during the vegetation period of 2009 and 2010. Results of linear-mixed effects models for predatory arthropods caught in salicylaldehyde-baited and non-baited traps. SA = salicylaldehyde, con = control, numDF = numerator degrees of freedom, denDF = denominator degrees of freedom. Bold font indicates significant *P*-values.

Family/Species	Year	SA [∑]	con [∑]	num DF	den DF	*F*-value	*P*-value
Cantharidae	2009	57	3	1	44	10.28	**0.0025**
	2010	31	2	1	51	21.75	< **0.0001**
Cantharis pellucida	2009	39	1	1	44	5.25	**0.0268**
	2010	11	0	1	51	6.84	**0.0117**
Rhagonycha sp.	2009	16	2	1	44	6.32	**0.0157**
	2010	20	2	1	51	10.07	**0.0026**
Coccinellidae	2009	18	3	1	44	9.72	**0.0032**
	2010	16	0	1	51	12.72	**0.0008**
Harmonia axyridis	2009	14	2	1	44	9.28	**0.0039**
	2010	14	0	1	51	10.06	**0.0026**
Panorpidae	2009	46	7	1	44	6.64	**0.0134**
	2010	45	5	1	51	5.81	**0.0196**

Experienced *Harmonia axyridis* adults caught in nature showed a significant preference to salicylaldehyde in the olfactometer test (Fig. 3). In contrast, the naïve specimens of *H. axyridis* were significantly deterred by salicylaldehyde (Fig. 3).

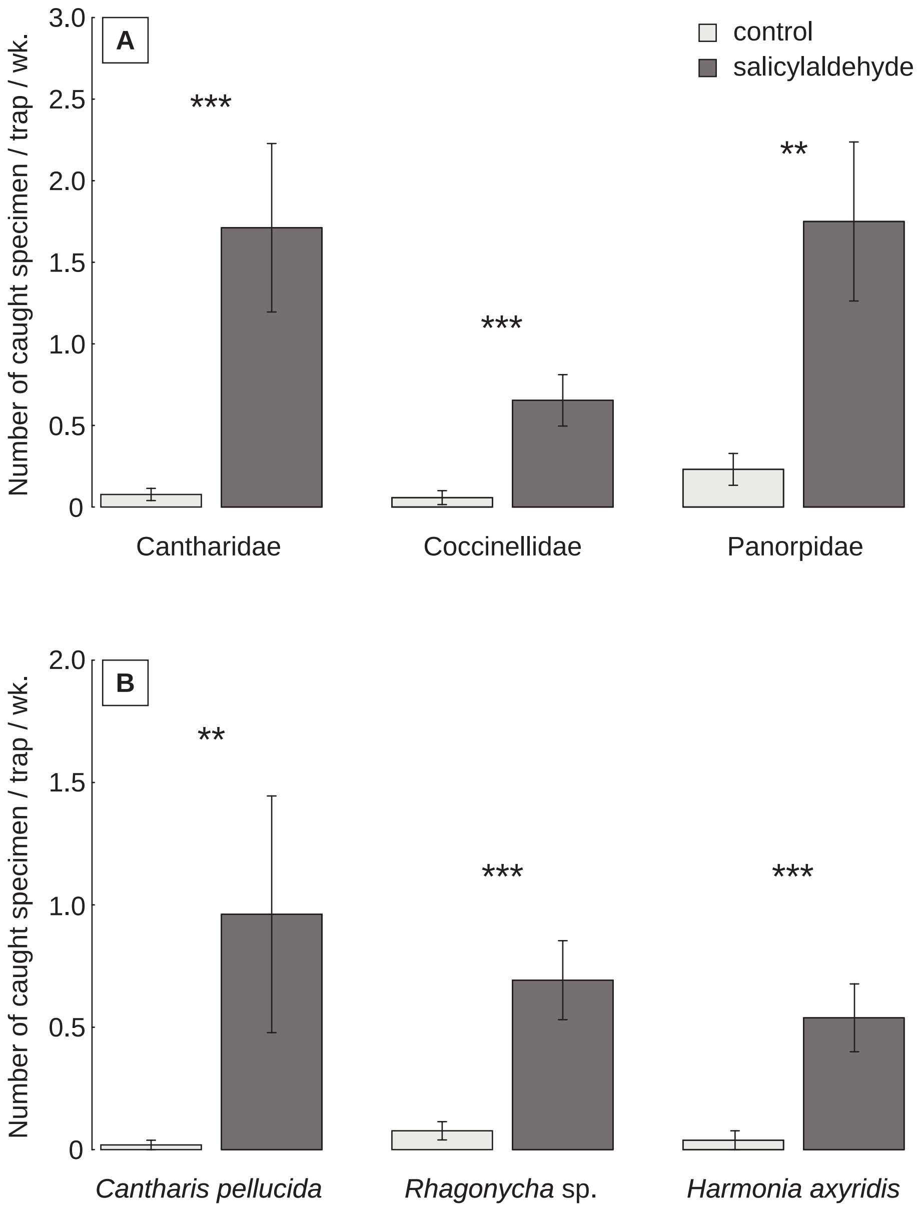

Figure 2: Weekly trap catches of different families (**A**) and the most abundant species (**B**) of generalist predators in response to the synthetic salicylaldehyde. Graphs show the combined data from 2009 and 2010. Mean ± standard error are given. ** $P < 0.01$, *** $P < 0.001$ (Linear Mixed Effects Model).

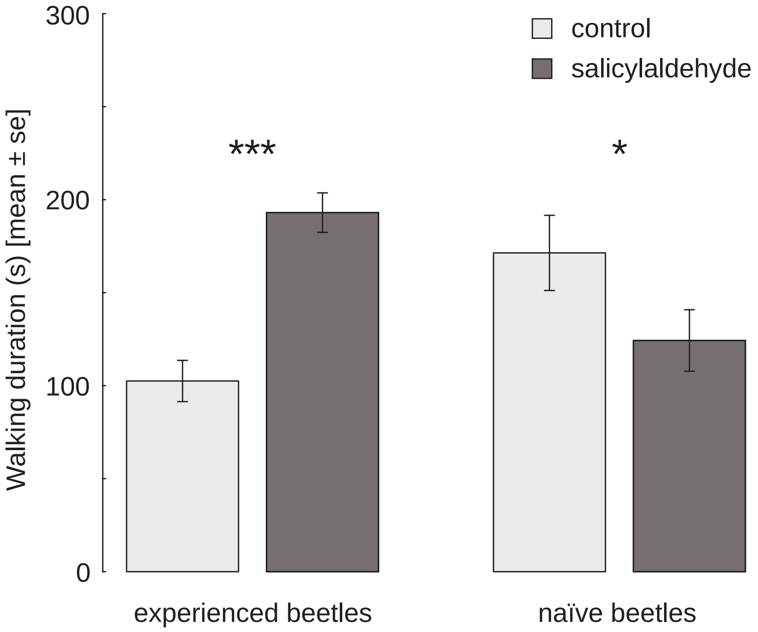

Figure 3: Walking duration time (mean ± standard error) of experienced and naïve *Harmonia axyridis* in the odor fields of an olfactometer. Experienced beetles N = 21, naïve beetles N = 20. ** $P < 0.01$, *** $P < 0.001$ (Wilcoxon-matched pairs test).

DISCUSSION

In this study, we investigated the response of generalist predator beetles to salicylaldehyde, the defense compound of leaf beetle larvae feeding on willow and poplar. Salicylaldehyde is sequestered from salicin, a phenolic glycoside that serves as feeding deterrent against generalist herbivores. Our results indicate that generalist predators were attracted by salicylaldehyde. This is in contrast to previous laboratory studies, where salicylaldehyde is described to deter generalist predators such as ants (Wallace and Blum, 1969, Kearsley and Whitham, 1992, Hilker and Schulz, 1994), spiders (Palokangas and Neuvonen, 1992), lady bird beetles (Denno et al., 1990, Smiley, 1991, Cha et al., 2009), as well as praying mantis (Cha et al., 2009).

The results of the field study and the laboratory experiment show an attraction of experienced *Harmonia axyridis* adults by salicylaldehyde. These results are in contrast to the study of Denno et al. (1990) and Smiley (1991). Both reported a repellent effect of salicylaldehyde to native lady bird beetles. However, our laboratory study with naïve *H. axyridis* showed a repellent effect of salicylaldehyde. This result supports the finding of Cha et al. (2009), who also proved the deterrence of salicylaldehyde with regard to *H. axyridis* larvae. Our results indicate that *H. axyridis* imagines seem to have a learning ability as Dejean et al. (2003) and Boivin et al. (2010) proved for lady bird beetle larvae. Schöller and Prozell (2002) described that the generalist egg parasite *Trichogramma evanescens* is able to learn the scent of possible hosts. They showed that experienced females response significantly to the main component of the sex pheromone of two Lepidoptera host species.

We suggest that the adults made an experience with salicylaldehyde as an indicator of prey availability. As additional ability *H. axyridis* have to overcome the defense system of salicin-using leaf beetle larvae. Like most other aphidophagous coccinellid beetles, *H. axyridis* is not a specialist predator. They also accept different lepidopteran and two chrysomelid species as prey (Tedders and Schaefer, 1994, Hodek and Honek, 1996, Koch et al., 2003). Koch et al. (2003) described the ability of *H. axyridis* to overcome the the defense of *Danaus plexippus* (Lepidotera) larvae. These larvae sequester cardenolides from their host plants that provide protection against predators (Seiber et al, 1980). One of the leaf beetle species that is reported to be hunted by *H. axyridis* is *Chrysomela vigintipunctata* that feeds on salicin rich willows and sequester salicylaldehyde in the defensive secretion (Soetens et al., 1998).

In our field study we found an attraction of soldier beetles *Cantharis pellucida* and beetles of the genus *Rhagonycha* to baited traps. To the best of our knowledge, there are no studies with Cantharidae analyzing olfactory attraction of defense substances of the host or of herbivore-induced plant volatiles. Cantharid adults were observed being carnivorous preying on aphids and other invertebrates (Sunderland et al., 1987) as well as being phytophagous consuming plant material (Gordon and Woodford, 1994). Traugott (2003) described adults of different *Cantharis* species mainly feeding on nectar, pollen and honeydew.

We did also not expect that *Panorpa* specimens were significantly more abundant in salicylaldehyde baited than in non-baited control traps. There is little evidence that *Panorpa* is an active predator of insects and their larvae, because they are mainly known to be scavengers, feeding on arthropod carrion (Thornhill, 1980). Therefore we assume that the *Panorpa* specimens were not attracted by the saliclyladhyde but by the scent of arthropod carrion in our funnel traps.

The results reported in this study indicate the potential of salicylaldehyde as an attractant for experienced generalist predators with respect to pest control of salicin-using leaf beetles and their larvae. These leaf beetles of the genera *Chrysomela*, especially *C. populi* and *C. tremulae* and *Phratora* sp. are reported to be the most important defoliating pests in young short rotation plantations in Central Europe (Sinreich, 1955, Augustin et al., 1993, Gruppe et al., 1999). Due to the increasing demand for renewable energy, short-rotation forestry with its highly productive *Salix* and *Populus* species becomes increasingly important as a potential energy source. Therefore, current ecological research should examine the biological control of such defoliating insect pests in more detail, e.g. the learning ability of *H. axyridis* imagines and larvae. Further, there may also be responses of *H. axyridis* and *Cantharis pellucida* to herbivore-induced volatiles and synergistic effects with salicylaldehyde that are still unknown.

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CHAPTER

5

POROUS DEFENSE IN A TRITROPHIC SYSTEM: ODOR PERCEPTION REFLECTS PREY SPECIALIZATION OF POTTER WASPS (HYMENOPTERA: EUMENINAE)

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submitted to *Journal of Chemical Ecology*

ABSTRACT

Predatory arthropods are attracted to infochemicals emitted by their herbivore prey or by herbivore-infested plants. We studied such a tritrophic system measuring the olfactoric responses of three potter wasp species (*Symmorphus murarius*, *Symmorphus gracilis*, *Discoelius zonalis*, Hymenoptera: Eumeninae), which differ in resource-use specialization, to herbivore-induced plant volatiles (HIPVs) emitted by aspen (*Populus tremula*) and saliclylaldehyde, sequestered as a defence compound by *Chrysomela* leaf beetle larvae. Electroantennographic recordings (GC-MS/EAG and EAG) exhibited differences in perception of HIPVs between the three wasp species. The highly specialized *S. murarius* (specialist on *Chrysomela*) was more sensitive to salicylaldehyde than the less specialized *S. gracilis*, feeding on leaf beetles and weevils, whereas *D. zonalis*, feeding on a range of microlepidopteran prey, did not respond at all. These results indicate that the sensory capability to perceive volatiles from prey and HIPVs can differ greatly according to the wasp species and its degree of resource specialization.

Key Words: electrophysiology, salicylaldehyde, *Symmorphus murarius*, *Symmorphus gracilis*, *Discoelius zonalis*, *Populus tremula*, herbivore-induced plant volatiles, HIPVs, (*Z*)-3-hexen-1-ol, Chrysomelinae

INTRODUCTION

Carnivorous enemies of herbivores exploit of several olfactoric strategies to sucessfully localize their prey. The use of plant volatiles, herbivore-induced plant volatiles (HIPVs) released by infested plants or directly of volatiles released by their prey such as pheromones and even allomones for the location of prey is known from many predatory insects (Dicke, 2009). In this way, such infochemicals often shape multitrophic interactions (Takabayashi and Dicke, 1996; Dicke and Baldwin, 2010). The use of infochemicals and especially HIPVs is known for a range of carnivores including parasites (Dougherty et al., 1999), parasitoids (Rutledge, 1996; Quicke, 1997) and predators such as predatory beetles (Herms et al., 1991; Yoneya et al., 2009), true bugs (Weissbecker et al., 2000) and wasps. The latter include solitary and eusocial species as Pompilidae (Punzo and Ludwig, 2005), Sphecidae (Anton and Gnatzy, 1998) and Vespidae (Aldrich et al., 1985; Hendrichs et al., 1994; Jander, 1998). To the best of our knowledge, no studies have been carried out on infochemical use by potter wasps (Eumeninae).

Females of solitary potter wasps prey upon insect larvae for provisioning their nests with food for their offspring. Most Eumeninae species prey on Lepidoptera larvae. *Discoelius zonalis* hunts on microlepidopteran larvae, wich in turn are herbivores of several plant species (Blüthgen, 1961). Other eumenid species forage for coleopteran larvae such as chrysomelids and curculionids (Blüthgen, 1961; Evans, 1966). Among those, *Symmorphus* species show different prey specializations on herbivores of Salicaceae (*Populus* and *Salix*). The highly specialized *S. murarius* exclusively hunts for Chrysomelinae leaf beetle larvae, whereas the less specialized *S. gracilis* accepts Chrysomelinae larvae and larvae of Curculionidae, especially of the genus *Cionus* (Blüthgen, 1961; Gathmann and Tscharntke, 1999; Budriene, 2003). *S. murarius* and *D.* zonalis are very rare in Central Europe, whereas *S. gracilis* is the most common species.

As an effective defence strategy, leaf beetle larvae feeding on Salicaceae sequester phenolic glucosides, especially salicin and its derivatives and transform it into salicylaldehyde (Pasteels et al., 1983; Smiley et al., 1985; Köpf et al., 1998; Burse et al., 2009). This salicylaldehyde is an effective deterrent and provides protection against several generalist arthropod predators such as ants (Wallace and Blum, 1969; Hilker and Schulz 1994), ladybird beetles (Denno et al., 1990; Smiley, 1991) and spiders (Palokangas and Neuvonen, 1992). In contrast, specialist predators adapted to the defence system are inviolable, and use salicylaldehyde as a kairomone for host finding. Those are phorid flies (Zvereva and Rank 2003, 2004), syrphid flies (Rank and Smiley, 1994; Köpf et al., 1997; Gross et al., 2004) and the sawfly *Tenthredo olivacea* (Pasteels and Gregoire, 1984).

Because of these differences in prey specialisation, our tritrophic model system consisting of *Populus tremula*, salicin-sequestering Chrysomelinae, and their predators, the potter wasps *S. muraurius* and *S. gracilis*, provide an interesting case to investigate how infochemicals shape the interactions in a comparative approach (Fig. 1).

The aim of this study was to determine whether there are differences in odor perception of *S. murarius, S. gracilis,* and *D. zonalis* to distinct HIPVs and salicylaldehyde, the allomone of the beetle larvae.

The questions adressed were:

1) Do the three wasp species perceive HIPVs?
2) Is the specialization degree of the three wasps to salicin-sequestering larve reflected in perception performance of salicyaldehyde?
3) In eumenid wasps brood provisioning is provided only by females. Do the males also detect plant odor and salicylaldehyde?

To answer these questions, GC-MS/EAD experiments were carried out to examine perception of HIPVs of *Populus tremula* by females of *S. murarius*, *S. gracilis and D. zonalis.* Comparative EAG dose-response experiments wer conducted to scrutinize the perception of salicylaldehyde, and selected HIPVs.

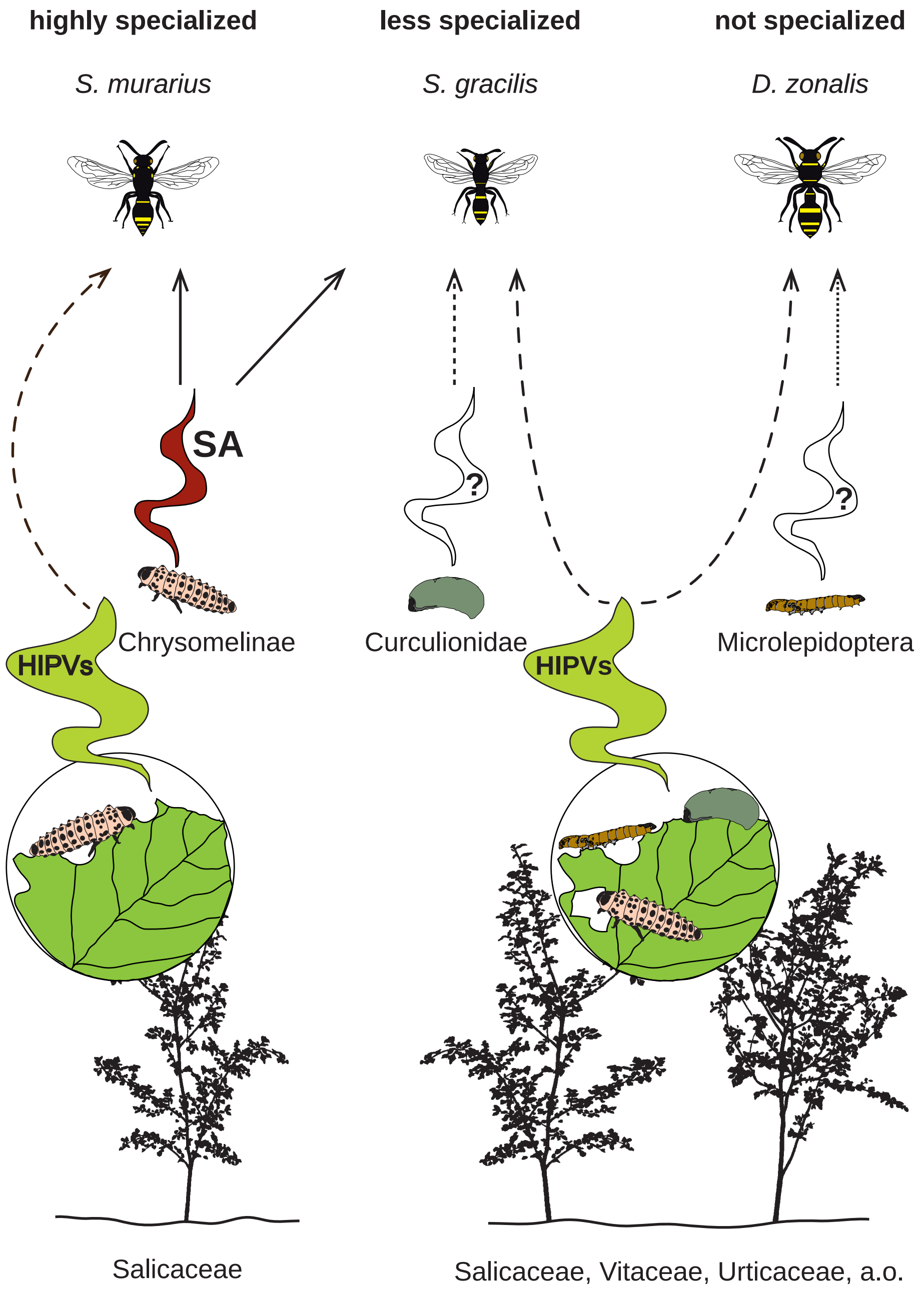

Figure 1: Schematic drawing of the tritrophic systems of Salicaceae, salicin-using leaf beetle larvae, and females of the potter wasps *Symmorphus murarius*, *S. gracilis*, and *Discoelius zonalis*. HIPVs = Herbivore-induced plant volatiles; SA = Salicylaldehyde. (Drawing of trees by W. Tambour, University of Göttingen).

MATERIAL AND METHODS

Insect Sampling

Specimens of the genus *Symmorphus* (*S. murarius* and *S. gracilis*) were obtained from artificial trap-nests consisting of plastic tubes of 15cm diameter filled with reed internodes (*Phragmites australis*) placed in the field from April to September 2008 in the vicinity of Göttingen, Lower Saxony, Germany. In total, 396 trap nests were installed. Additional specimens of *D. zonalis* were obtained from numerous trap-nests placed 2009 near Baderitz (Sornzig-Ablaß), Saxonia, Germany. During winter 2008 and 2009, all traps were opened in the laboratory and examined for occupied reed internodes (Gathmann and Tscharntke, 1999). All occupied reeds were stored at 2 – 6 °C in a climate chamber until eclosion of adult individuals in early spring. In total only six *S. murarius* (3 females and 3 males), 55 *S. gracilis* (20 females and 25 males), and one *D. zonalis* (male) hatched in 2009. In 2010, another 22 *D. zonalis* (12 females and 10 males) hatched. Number of hatched individuals reflects the commonness of the respective species. Wasps were identified to species level using the identification keys of Blüthgen (1961) and Schmid-Egger (1994).

Volatile Sampling and Extracts

Volatiles were collected from naturally herbivore-infested eight year old poplar trees (*Populus tremula*) on an experimental field in July 2008. The trees were part of a biodiversity experiment carried out in 2008 as described by Kleemann et al. (2010). Volatiles were sampled on a charcoal trap (CLSA-Filter, Daumazan sur Arize, France) using a modified push-pull headspace collection system (Tholl et al., 2006) directly on the trees. Always about 50 leaves of a branch were enclosed in a plastic roasting bag (Melitta GmbH, Minden, Germany). Air was circulated through the trap by a miniature pump (Fürgut, Aichstetten, Germany) at a flow of 0.8 l min^{-1}. The sampling time was 2 hr. Adsorbed volatiles were eluted with 100 µl of dichloromethane/methanol (2:1). The solvents used were of analytical quality (Suprasolv quality, Merck/VWR, Darmstadt, Germany). After elution, samples were stored in an ultralow temperature freezer at -80°C.

Chemicals

The following authentic standards were obtained with given purity from commercial sources: 6-methyl-5-hepten-2-one (96%, CAS: 110-93-0, Fluka, Germany), (*Z*)-3-hexen-1-ol (98%, CAS: 928-96-1, Merck-Suchardt, Hohenbrunn, Germany), (*Z*)-3-hexenyl acetate (98%, CAS: 3681-71-8, Aldrich, Germany), linalool (97%, CAS: 78-70-6, Merck, Germany), (*E/Z*)-linalool oxide (97%, CAS: 60047-17-8, Acros, Germany), methyl salicylate (99%, CAS: 119-36-8, Sigma-Aldrich, Germany), β-caryophyllene (98.5% CAS: 87-44-5, Sigma-Aldrich, Germany), nonanal (98%, CAS: 124-19-6, Merck, Germany), ocimene (mixture 70% (Z)-ocimene + 25% limonene, 98%, CAS: 13877-91-3, Fluka, Germany), and salicylaldehyde (99%, CAS: 17754-90-4, Aldrich, Steinheim, Germany).

GC-MS/EAD System and Data Analyses

Volatile samples were analyzed with a coupled GC-MS/EAD system (Weissbecker et al., 2004) consisting of a gas chromatograph Agilent type 6890 connected to a type 5973 quadrupole mass spectrometer (both Palo Alto, USA) with electron ionisation (EI, 70 eV), connected to an “olfactory detector port” (ODP-2, Gerstel, Mülheim, Germany) and custom made antennae holder equipped with an EAG-amplifier (Dr. Koch, University of Kaiserslautern). Two column types in a similar setup, a HP-INNOWax (Agilent, 30 m, 0.25 mm ID, 0.25 µm coating thickness, polyethyleneglycol), and a HP-5ms (Agilent, 30 m, 0.25 mm ID, and 0.25 µm film thickness, phenylmethylsiloxane) were used to validate the composition of the extracts. An aliquot of 1 µl was injected into the injector held at 250°C. The oven temperature program was 50°C held for 1.5 min, followed by an increase of 7.50 °C/min to 200°C, remaining at 200°C for 5 min. Helium (purity 99.999 %) was used as the carrier gas (1 ml/min). The effluent from the column was splitted (1:1) into two pieces of deactivated capillary using a Graphpack 3D/2 flow splitter (Gerstel, Mülheim, Germany). One capillary (1m × 0.1mm i.d.) led to the mass spectrometer, the other (1m × 0.15mm i.d.) to an “olfactory detector port” (ODP-2, Gerstel). Volatiles were guided through the capillary into the EAD-interface and were mixed with humidified air (23°C, 80% RH) with a flow rate of 400 ml/min.

Excised antennae of both sexes of the three potter wasp species were placed into an antenna holder (Färbert et al., 1997), where both antenna ends contacted an electrolyte solution for providing electrical contact to a pair of Ag/AgCl electrodes. We used an amplification factor of 100 for the EAD potentials generated by high-impedance amplifier (input impedance 100 MΩ; Prof. Koch, Kaiserslautern, Germany). After digitization (35900E A/D converter, Agilent) the signals were recorded with the GC ChemStation software (D.02.00.275, Agilent Technologies). For identification of the constituents, mass spectra and GC linear retention indices (van den Dool and Kratz, 1963) were compared to those of authentic standards and those of the mass spectral databases Wiley 9 combined with NIST ´08 (McLafferty & Hoboken, 2009).

Electroantennographic Recordings

Dose-response experiments were conducted with antenna of females and males of the three eumenid species using an EAG setup as described in Weissbecker et al. (2004). The series were carried out by manually injecting the synthetic volatile standards upstream the dissected antenna into a stream with synthetic air. To guarantee standard conditions stimuli were supplied every 120 s. Dilutions of synthetic standards were prepared of (*Z*)-3-hexen-1-ol, nonanal, and salicylaldehyde in paraffin oil (Uvasol®, spectrosc. qual., high visc., Merck, Darmstadt, Germany) in doses from 10^{-5}, 10^{-4}, 10^{-3}, and 10^{-2} (w/w) in order to calculate a dose-response curve. Ca. 100 µl of standard dilution or paraffin oil as a control were dropped on 2 cm^2 filter paper pieces (Schleicher & Schuell, Dassel, Germany). A soaked filter paper was inserted into a 10 ml glass syringe (Poulten & Graf GmbH, Wertheim, Germany). A typical stimulus was supplied by puffing 5 ml of air over the antenna (Schütz et al., 1997) and repeated once for each concentration and control. The EAG response for each dilution and control was recorded for *S. murarius*, *S. gracilis* and *D. zonalis*.

Statistical Analyses

Statistical analyses were carried out using the software R, Version 2.10.0 (R Development Core Team, 2010). We subtracted the mV-response to the initial paraffin control from all obtained compound responses. Thus we obtained data for four doses to describe a dose-response relationship. We refrained from normalizing the EAG response data. To analyze the EAG response we used a non-linear regression approach using the software package “drc” generally designed for the analysis of multiple dose-response curves (Ritz and Streibig, 2005). We fitted dose-response curves with three-, four- and five-parameter logisitic non-linear models. To select the minimal adequate model we used the one with the lowest Akaike´s Information Criterion (AIC) (Burnham and Anderson, 2002). Sensitivity parameters have been calculated for comparison between species and compounds. We selected the ED_{10} (effective dose evoking 10% of the total response), as an estimate value of the odor detection threshold. The limit of detection according to IUPAC definitions (IUPAC Compendium of Chemical Terminology, 2nd edition 1997) has been calculated for blank measures of selected antennae. ED_{10} was always above those values. Additionally we calculated the ED_{50}, which characterizes the point of steepest slope of the curve, and calculated the detection range (ED_{50} – ED_{10}) (see van Giessen et al., 1994). An overview of the use of non-linear curve fitting and interpretation of calculated sensitivity parameters in insect chemoreception is given by van Giessen et al. (1994) and Jordan (2009). To calculate differences between paraffin oil control and the lowest concentration of synthetic compounds we used a two sample *t-test*.

Differences between responses of females and males to the 10^{-3} concentration were considered significant at a *P*-value < 0.05 (*ANOVA F-test*). Data were tested for normality with the *Shapiro-Wilk's test*. If required, data were log-transformed to meet the assumptions of homogeneity of variance and normality of residuals of the statistical models.

RESULTS

GC-MS/EAD Recordings

The three species showed antennal responses to different compounds of poplar volatile extracts, i.e. alcohols, aldehydes, ketones, (acyclic) monoterpenes, and sesquiterpenes. Reproducable antennal responses of all three wasp species could be verified for (*Z*)-3-hexen-1-ol, (*Z*)-3-hexenyl acetate, 6-methyl-5-hepten-2-one, (*E*/*Z*)-linalool oxide and β-caryophyllene. Methyl salicylate was perceived by the two *Symmorphus* species only. Linalool evoked antennal responses in *S. murarius* and *D. zonalis*, ocimene exclusively in *S. gracilis* (Table 1).

Table 1: Electroantennogam activity from antennae of *Symmorphus murarius*, *S. gracilis*, and *Discoelius zonalis* to different compounds in plant volatile extracts from *Populus tremula* leaves.

Compound	*S .murarius*	*S. gracilis*	*D. zonalis*
6-methyl-5-hepten-2-one	++	+	+++
(*Z*)-3-Hexen-1-ol	++	+	++
(*Z*)-3-Hexenyl acetate	++	+	++
Linalool	++	-	++
(*E*/*Z*)-Linalool oxide [a]	+	+	+
Methyl salicylate	++	+	-
β-Caryophyllene	+	+	+
Nonanal *	NA	+	-
Ocimene [a]	-	+	-

[a]: Stereochemistry not determined. * was not present in all extracts. Response categories: 0-0.5 mV (-), 0.5-5 mV (+), 5-15 mV (++), 15-25 mV (+++)

Electroantennographic Experiments

Antennae of all three eumenid wasps responded to (*Z*)-3-hexen-1-ol resulting in a similar detection threshold (ED_{10}). *S. murarius* had the broadest detection range for this plant volatile and among those tested it was the only one perceived by *D. zonalis* (Table 2, Fig. 2). Both *Symmorphus* species perceived nonanal and had a similar detection threshold, whereof *S. gracilis* showed the broadest detection range. The antennae of *S. murarius* females were exceedingly sensitive to salicylaldehyde (Fig. 2 and Appendix Table A1). The detection

Table 2: Estimated sensitivity parameters derived from fitted dose-response curves of electroantennogram activities from antennae of *Symmorphus murarius*, *S. gracilis*, and *Discoelius zonalis* to different concentrations of salicylaldehyde, nonanal and (*Z*)-3-hexen-1-ol. ED10 = detection threshold, ED50 = detection half response, ED50 - ED10 = detection range. *S. murarius* N = 3, *S. gracilis* N = 11, *D. zonalis* N = 8. conc = concentration. SE = standard error.

		Threshold	Half reponse	Detection range
		ED10	ED50	ED50 ~ ED10
Compound	Species	log10 conc. ± SE	log10 conc. ± SE	log10 conc. ± SE
Salicylaldehyde	*S. murarius*	-7.6 ± 0.8	-5.3 ± 0.5	2.3
	S. gracilis	-2.7 ± 0.8	-1.3 ± 0.9	1.4
	D. zonalis	no response	no response	no response
Nonanal	*S. murarius*	-4.4 ± 0.4	-2.7 ± 1.3	1.8
	S. gracilis	-4.8 ± 0.5	-1.5 ± 0.7	3.3
	D. zonalis	no response	no response	no response
(*Z*)-3-hexen-1-ol	*S. murarius*	-3.6 ± 0.3	-1.7 ± 0.6	1.9
	S. gracilis	-3.6 ± 0.1	-2.6 ± 0.5	1.0
	D. zonalis	-3.2 ± 0.5	-1.6 ± 0.7	1.6

threshold (ED_{10}) of salicylaldehyde is about 3 magnitudes lower compared to nonanal and 4 magnitudes lower compared to (*Z*)-3-hexen-1-ol (Table 2). The analyses of sensitivity parameters revealed that *S. murarius* was much more sensitive to salicylaldehyde than *S. gracilis* (i.e. about 5 magnitudes lower $ED_{10,}$ and 4 magnitudes lower ED_{50}), consequently it also showed a broader detection range (ED_{50} - ED_{10}) (Table 2). *S. gracilis* perceived salicylaldehyde, but antennae were less sensitive to it than to the other two compounds. There was a significant difference between paraffin oil controls and the lowest concentration tested (10^{-5}) of salicylaldehyde for *S. murarius* only (df = 4, *t*-value = -6.7246, *P*-value = 0.0025). In most cases, the females had a higher sensitivity to the three tested compounds than the males, albeit not always being significant. Nevertheless, all substances were perceived by the male wasps (Table 3).

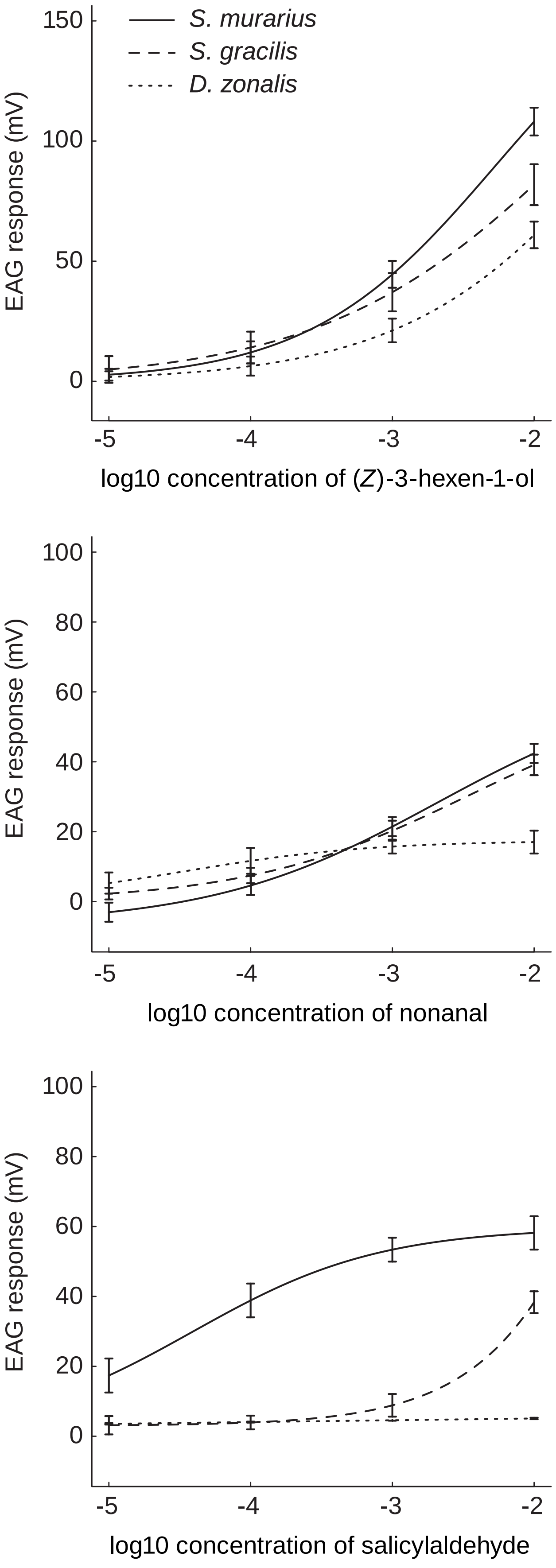

Figure 2: Dose-response curves (EAG) of females of the three potter wasp species *Symmorphus murarius*, *S. gracilis*, and *Discoelius zonalis*. Error bars show the SE of the fitted logistic curves. Mean and SE values of measured antennal response is depicted in Table 2. Doses are shown as log10 concentrations in paraffin (w/w). Responses are absolute amplified values [mV]. *S. murarius* N = 3, *S. gracilis* N = 11, *D. zonalis* N = 8.

Table 3: EAG response comparison of females and males of *Symmorphus murarius*, *S. gracilis*, and *Discoelius zonalis* to a 10-3 concentration of salicylaldehyde, nonanal, and (*Z*)-3-hexen-1-ol. Data are given with mean and standard errors (SE). n.s. = $P > 0.05$, * = $P < 0.05$, ** = $P < 0.01$.

			Salicylaldehyde				Nonanal				(*Z*)-3-hexen-1-ol			
Species	Sex	n	mean		SE		mean		SE		mean		SE	
S. murarius	♀♀	3	50.00	±	5.99	**	21.45	±	2.72	*	44.00	±	5.33	n.s.
	♂♂	3	19.54	±	0.84		7.97	±	2.76		18.56	±	10.72	
S. gracilis	♀♀	11	11.77	±	2.31	n.s.	22.45	±	3.44	*	39.34	±	7.87	n.s.
	♂♂	5	11.84	±	2.44		9.14	±	2.22		34.62	±	6.86	
D. zonalis	♀♀	8	6.78	±	1.95	n.s.	13.20	±	3.93	n.s.	24.28	±	3.62	n.s.
	♂♂	3	2.41	±	1.90		12.24	±	1.12		15.37	±	3.61	

DISCUSSION

Our results show that the potter wasps *S. murarius*, *S. gracilis* and *D. zonalis* perceive terpenoids, green leaf volatiles and aromatic compounds, major classes of HIPVs. *S. murarius* antennae were much more sensitive to salicylaldehyde than *S. gracilis*, whereas *D. zonalis* was not able to detect salicylaldehyde at all. We assume that this reflects degree of prey specialization of these species. *Symmorphus* species are specialists of salicin-sequestering Chrysomelinae (Blüthgen, 1961; Rank, 1994). *S. murarius*, showing strongest olfactory sensitivity to salicylaldehyde, exclusively hunts salicin-sequestering *Chrysomela* larvae, whereas *S gracilis* hunts also *Cionus* larvae (Curculionidae) (Blüthgen, 1961; Gathmann and Tscharntke, 1999; Budriene, 2003). Salicin sequestration is not known in Curculionidae.

Depending on salicin content of Salicaceae hosts, most species of the genus *Chrysomela* sequester salicin into salicylaldehyde (Pasteels et al., 1990; Termonia et al., 2001). Salicylaldehyde is an effective deterrent and provides protection against several generalist arthropod predators (Denno et al., 1990; Hilker and Schulz 1994) except *Symmorphus* species. These specialists have adapted and overcome the defence of Chrysomelinae. We suggest that *Symmorphus* species use the allomone salicylaldehyde as a kairomone for host finding. All European *Symmorphus* species (*S. allobrogus, S. angustatus, S. bifasciatus, S. connexus, S. crassicornis, S. cristatus*) including *S. gracilis* and *S. murarius* were found to prey primarily salicin-sequestering Chrysomelinae larvae (Blüthgen, 1961; Gathmann and Tscharntke, 1999; Budriene, 2003). Exceptions are *S. gracilis* and *S. connexus*, additionally hunting for larvae of Microlepidoptera and Curculionidae. *D. zonalis* is a generalist preying larvae of different Microlepidoptera species. They neither prey on *Chrysomela* leaf beetles, nor is anything reported about salicylaldehyde in the defence of Microlepidoptera larvae. Therefore, we suspect that *D. zonalis* has no antennal receptors, sensitive to salicylaldehyde. The North American potter wasp species *Symmorphus cristatus* is also a specialist of salicin-sequestering leaf beetle larvae (Rank, 1994; Sears et al., 2001) and Sears et al. (2001) were the first who, suspected that *Symmorphus cristatus* use salicylaldehyde, the allomone of their prey, for host finding. To the best of our knowledge, our study is the first proving that *Symmorphus* wasps, specialized predators of salicin-sequestering leaf beetle larvae, perceive salicylaldehyde.

All three wasp species showed a similar perception ability to plant volatiles considered as herbivore induced. Among those, green leave volatiles (GLV), such as (*Z*)-3-hexen-1-ol and (*Z*)-3-hexenyl acetate as well as terpenoids, as 6-methyl-5-hepten-2-one, (*E*/*Z*)-linalool oxide and β-caryophyllene are perceived by all three species. In contrast nonanal is hardly perceived by *D. zonalis*. The aromatic methyl salicylate evoked, just like salicylaldehyde, antennal responses only in both *Symmorphus* species. The chemical structure is very similar to salicylaldehyde and it could be possible that both compounds activate the same olfactoric receptor. This could explain why *D. zonalis* does not perceive methyl salicylate, albeit it is considered as a HIPV (Dickens, 1999; de Boer and Dicke, 2004; Dicke, 2009). Antennal activity and behavioral attractiveness of HIPVs for carnivorous arthropods has been shown in numerous studies (Turlings et al., 1990; Dicke and Vet, 1998; Dickens, 1999; Steidle and van Loon, 2003; Dicke and Baldwin, 2010) and it is assumed that this is a widespread and advantageous way to locate their prey (Dicke, 2009). Eusocial wasps, *Vespula maculifrons, Polistes arizonesis* and *Mischocyttarus flavitarsis* (Vespidae) have been discussed to use host plant chemicals as foraging kairomones (Aldrich et al., 1985; Cornelius, 1993; Geitzenauer and Bernays, 1996). Only few studies of predators concerning HIPVs applied electroantennographical methods. Stinkbugs, (Pentatomidae, Heteroptera), predators of *Leptinotarsa decemlineata*, evoke antennal responses to HIPVs produced by potato herbivory (Dickens, 1999; Weissbecker et al., 2000).

Males of all three eumenid wasp species perceive salicylaldehyde, nonanal and (*Z*)-3-hexen-1-ol in most cases similar to females. However, *Symmorphus* males hardly responded to nonanal. *S. gracilis* males had a comparably high sensitivity to salicylaldehyde as the females, albeit not participating in nest provisioning. Males could be observed in the field flying around *Chrysomela* larvae-infested poplar trees searching and waiting for females. It could be speculated, that males use salicylaldehyde alone or in combination with HIPVs, such as (*Z*)-3-hexen-1-ol as a sexual kairomone. Sexual kairomone use is known for the cockchafers *Melolontha melolontha* and *M. hippocastani* (Reinecke et al., 2002; Ruther et al., 2002).

Our results showed that *S. murarius* and *S. gracilis* perceive the defence compound salicylaldehyde from salicin-sequestering leaf beetle larvae (Chrysomelinae) and further odor compounds released by *P. tremula* trees, among them many HIPVs. In contrast, the little specialised species *D. zonalis* detected only HIPVs and showed no perception ability to salicylaldehde. We conclude that salicylaldehyde is a major kairomone for host finding in those *Symmorphus* species, preying on salicin-sequestering Chrysomelinae, yet to be confirmed in behavioural studies.

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APPENDIX

Table A1: Non-normalized values of electroantennogram activities from antennae of *Symmorphus murarius*, *S. gracilis*, and *Discoelius zonalis* to different concentrations of salicylaldehyde, nonanal and (*Z*)-3-hexen-1-ol. Data are given with mean and standard errors (SE). *S. murarius* N = 3, *S. gracilis* N = 11, *D. zonalis* N = 8.

		S. murarius			*S. gracilis*			*D. zonalis*		
Compound	Concentration	mean	±	SE	mean	±	SE	mean	±	SE
Salicylaldehyde	10^-5	16.30	±	1.91	1.63	±	1.77	1.90	±	1.58
	10^-4	41.01	±	4.56	6.17	±	1.86	2.50	±	2.10
	10^-3	50.00	±	5.99	11.77	±	2.31	6.78	±	1.95
	10^-2	60.14	±	6.18	41.24	±	5.31	4.09	±	5.39
Nonanal	10^-5	-3.03	±	1.96	-0.27	±	1.20	4.09	±	2.64
	10^-4	4.60	±	1.15	10.44	±	2.19	12.75	±	2.52
	10^-3	21.45	±	2.72	22.45	±	3.44	13.20	±	3.93
	10^-2	42.39	±	4.15	42.74	±	4.31	17.84	±	2.40
(*Z*)-3-hexen-1-ol	10^-5	0.04	±	1.35	7.23	±	2.38	7.12	±	2.20
	10^-4	13.80	±	2.20	11.84	±	2.21	4.19	±	1.79
	10^-3	44.00	±	5.33	39.34	±	7.87	24.28	±	3.62
	10^-2	108.19	±	10.50	94.62	±	17.27	68.12	±	7.82

CHAPTER

6

SYNTHESIS

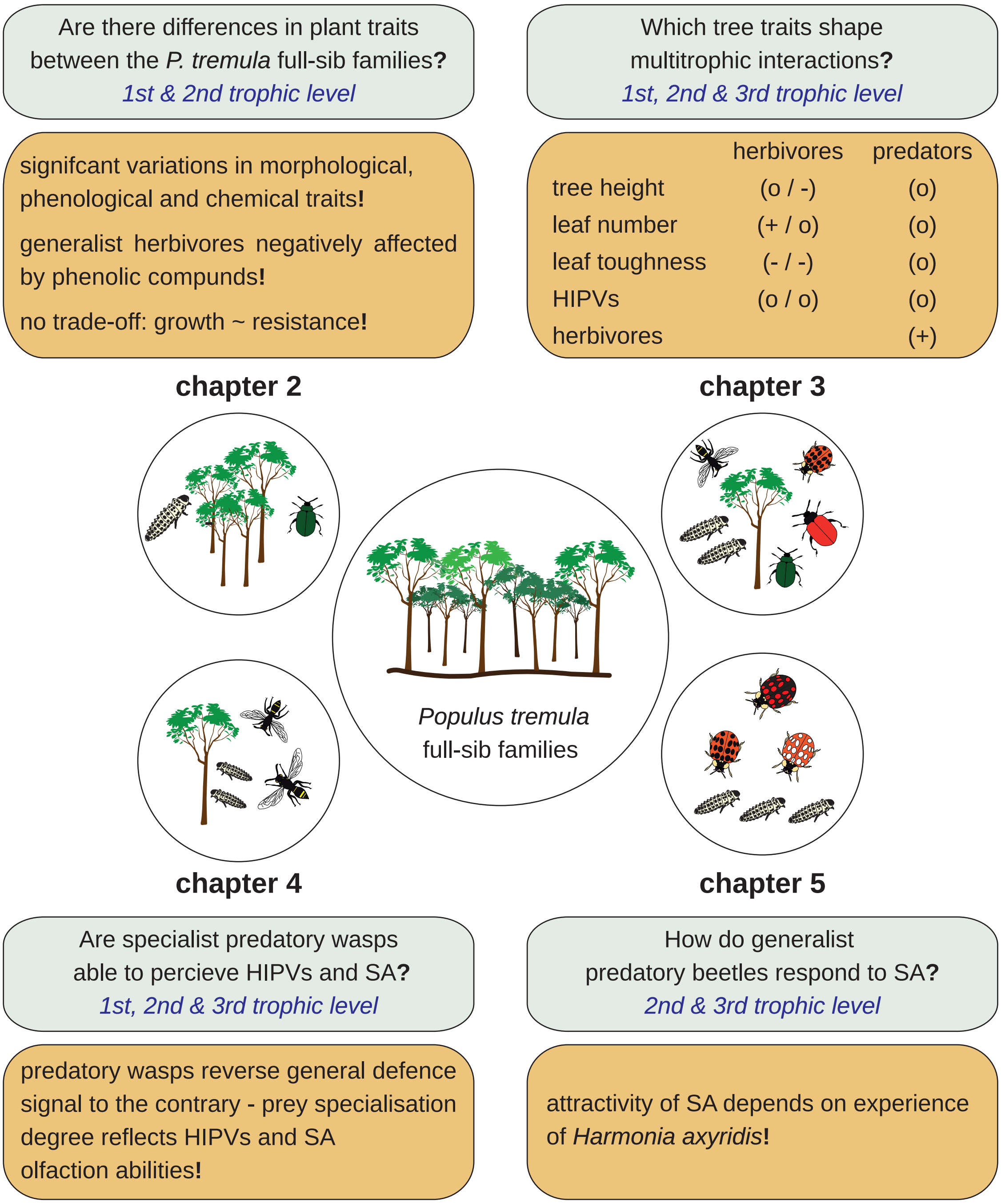
SYNTHESIS

Research on interactions of willows and poplars with herbivorous insects focuses mostly on the role of leaf chemical ecology using *Salix* clones or *Populus* hybrids, whereas less is known from real-world tritrophic interactions on genetically different *Populus tremula* trees.

In this thesis we used field observations to identify the community composition and abundance of herbivorous insects and predatory arthropods of seven closely related aspen (*P. tremula*) full-sib families. As observational studies are often criticized by lacking control and manipulation of possible confounding variables, we analyzed different morphological and chemical tree traits and used experimental methods (e.g. electroantennography) to investigate tritrophic interactions between poplar trees, chewing herbivores and their natural enemies (Fig. 1).

In our study the variation of neutral molecular genetic markers (nuclear microsatellites) was higher within each family than between the families. It was not possible to relate the different phenotypic characteristics and ecological traits (e.g. herbivore load) to the genetic variation based on molecular markers, because of the small number (only five) of neutral markers.

On the first trophic level we investigated aspen (*P. tremula*) of seven full-sib families. They differed in morphological traits such as leaf number, tree height and biomass production as well as in phenolic defensive compounds and other phytochemical leaf traits, whereas there were no differences in leaf toughness and herbivore-induced plant volatiles. The biomass production was closely related to the variation in plant morphological traits (e.g. leaf number, leaf area, etc.), but not to leaf chemistry. This is in contrast to the recent literature where the trade-off between chemical defence and growth is frequently discussed. Costs for increased defence can reduce plant growth (Simms and Rausher, 1987), whereas a decrease of defence can reduce fitness and growth if herbivores are present (Philippe and Bohlmann, 2007). In general, high levels of functional trait variations could be related to ecosystem stability in other studies (Lecerf and Chauvet, 2008).



Are there differences in plant traits between the *P. tremula* full-sib families**?**
1st & 2nd trophic level

signifcant variations in morphological, phenological and chemical traits**!**

generalist herbivores negatively affected by phenolic compunds**!**

no trade-off: growth ~ resistance**!**

Which tree traits shape multitrophic interactions**?**
1st, 2nd & 3rd trophic level

	herbivores	predators
tree height	(o / -)	(o)
leaf number	(+ / o)	(o)
leaf toughness	(- / -)	(o)
HIPVs	(o / o)	(o)
herbivores		(+)

Are specialist predatory wasps able to percieve HIPVs and SA**?**
1st, 2nd & 3rd trophic level

predatory wasps reverse general defence signal to the contrary - prey specialisation degree reflects HIPVs and SA olfaction abilities**!**

How do generalist predatory beetles respond to SA**?**
2nd & 3rd trophic level

attractivity of SA depends on experience of *Harmonia axyridis***!**

Figure 1: Scheme of the study objectives with the main questions of each chapter of the thesis (green rectangle) and the most important results (orange rectangle). HIPVs = herbivore-induced plant volatiles, SA = salicylaldehyde, (+) = positive effect, (-) = negative effect, (o) = no effect. Chapter 3: (+ / +) the first part within the bracket = influence on leaf beetle adults, the second part = influence on leaf beetle larvae.

In both observational studies (chapter 2 & 3) specialised leaf beetles from the genera *Phratora* and *Chrysomela* and their larvae were the most abundant specimen of the second trophic level (chewing herbivores). Their abundances on the trees were influenced by morphological traits such as tree height, leaf number, total leaf area, and leaf toughness as well as by chemical traits (phenolic compounds). Our analyses indicate that the phenolic compounds had the strongest effect on the herbivores (chapter 2) (Fig. 2). These results are in line with the recent literature (Lindroth and Bloomer, 1991, Matsuki and MacLean, 1994, Hemming and Lindroth, 1995, Peacock et al., 1999, Boeckler et al., 2011). Additionally we observed a spatio-temporal shift of herbivores and predators within our field site (chapter 3). This spatial distribution across the poplar field site suggested colonization from adjacent hedges, because in spring leaf beetles were most abundant in the field half close to the hedges. In contrast, highest abundances in summer were found in the second half of the field, presumably due to avoidance of tree leaves with induced responses to high beetle density in spring.

The most abundant predatory arthropods in the second experimental field (2009) were generalist predators such as coccinellid and cantharid beetles and spiders (third trophic level). Only few specialised predators such as *Symmorphus gracilis* (Hymenoptera, Eumenidae) could be observed. The analyses revealed that predatory arthropods were positively affected by the abundance of the leaf beetles, their larvae and eggs. Moreover, we could show that the generalistic coccinellid and cantharid beetles used salicylaldehyde as attractant. Our results further suggest that *Harmonia axyridis* imagines are able to learn to use salicylaldehyde as an attractant. This is in contrast to the recent literature as salicylaldehyde sequestered from salicin and its derivatives is known to provide protection for leaf beetle larvae against generalist predators (Wallace and Blum, 1969, Smiley, 1991, Palokangas and Neuvonen, 1992).

Similar to the generalist predators in our study the defence effect of salicylaldehyde was ineffective against specialist predators (Pasteels and Gregoire, 1984, Smiley and Rank, 1986, Rank and Smiley, 1994, Zvereva and Rank, 2003). This indicates that specialist predatory eumenid wasps are able to perceive salicylaldehyde and herbivore-induced plant volatiles and that the olfaction ability reflects the degree of prey specialisation of different wasp species. Our results suggest the use of infochemicals for host location and finding by eumenid wasp species (Fig. 2).

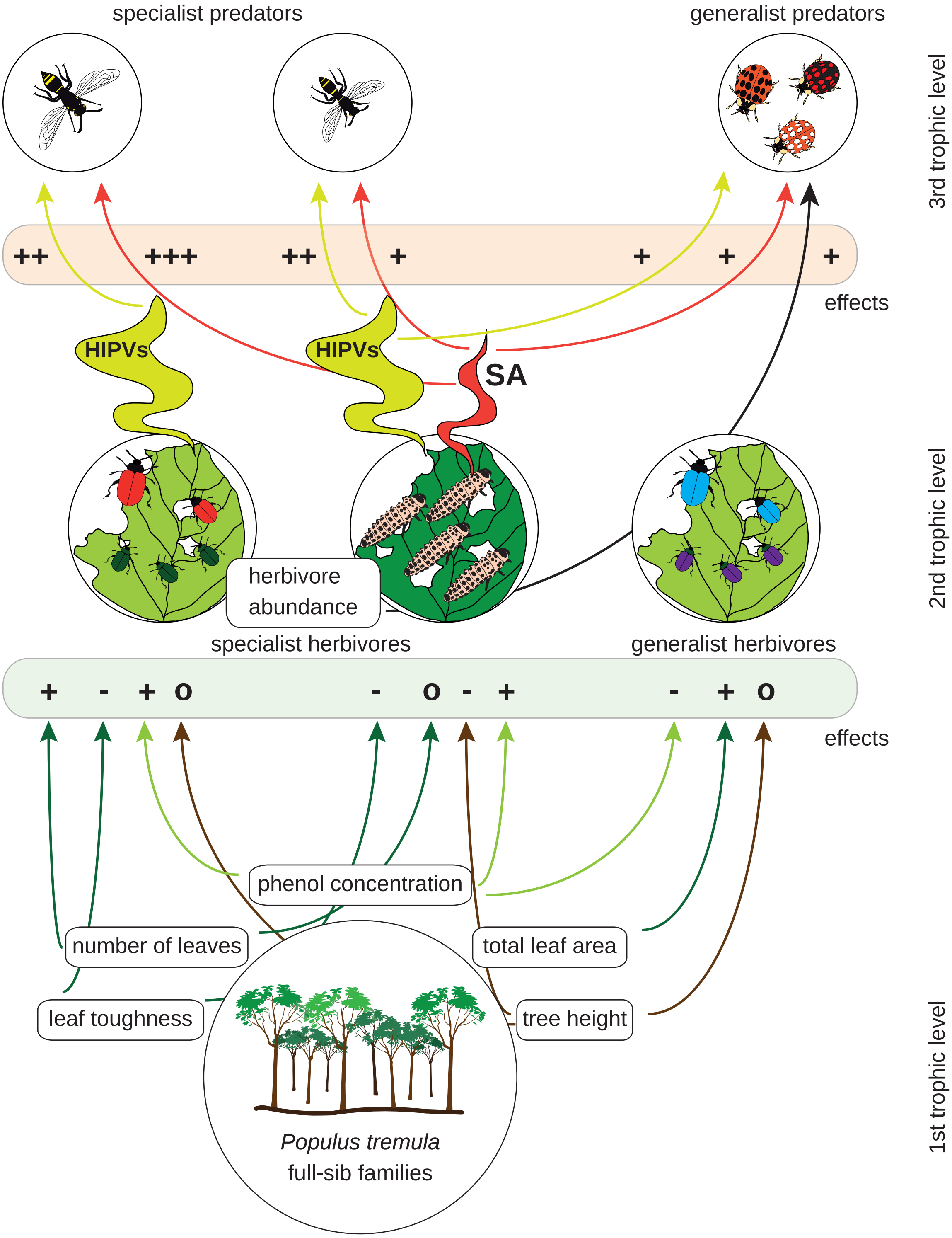


Figure 2: Synthesis of the results of the thesis. HIPVs = herbivore-induced plant volatiles, SA = salicylaldehyde, (+) = positive effect, (-) = negative effect, (o) = no effect. Specialist predatory wasps: (+), (++) and (+++) = differences in olfaction ability reflects the degree of prey specialisation of different specialized wasp species.

Overall we conclude that direct and indirect effects shape aspen-insect interactions. Aspen influence herbivorous insects directly via plant morphological, chemical and genetic traits as well as indirectly via their spatial distribution and via temporal aspects. Predators are likewise directly influenced by aspen via emitted herbivore-induced plant volatiles and indirectly via herbivore abundances on the trees. Furthermore, infochemicals emitted by their prey affect generalist and specialist predators in the tritrophic system consisting of *Populus tremula*, specialist leaf beetles, their larvae and their predators.

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SUMMARY

SUMMARY

A drastic increase of global energy consumption is expected for the following years. Due to the increasing demand for renewable energy, much of the current research focuses on short-rotation forestry with high productive *Salix* and *Populus* species as a potential energy source. In this context the European aspen (*Populus tremula*) gained importance for biomass production, because of its ability to reach considerable growth rates even on poor soils and under unfavourable habitat conditions. The European aspen is a pioneer species and is one of the most widespread tree species in boreal and temperate forests. On natural sites aspen create habitats for a rich arthropod community including many endangered species. Current knowledge in plant-insect interactions of willows and poplars is based on studies, which are mostly concentrated on only two trophic levels and differences in leaf chemistry affecting phytophagous insects.

The aim of the first part of this thesis was to characterize the differences between closely related aspen (*P. tremula*) full-sib families and to examine the influence of their morphological and chemical traits on herbivorous insects and their natural enemies in real-world multitrophic interactions. Thereto two common garden experiments were established with closely related aspen full-sib families (6 - 51 % genetic distance).

As hypothesized the aspen full-sib families differed in morphological traits such as tree height and leaf number as well as in leaf chemical traits such as phenolic glycosides. In the first common garden experiment a negative relationship was found between phenolic glycosides and chewing herbivorous insects (e.g. *Phratora vulgatissima*). In the second common garden experiment specialist leaf beetles and their larvae were influenced by different morphological tree traits. The imagines were positively related to the number of leaves (i.e. the resource abundance), whereas leaf beetle larvae were negatively related to tree height, which is in contrast to Lawton's plant size hypothesis. Herbivore-induced plant volatiles differed between spring and summer, but did not change with aspen genetics or herbivore load. Generalist predatory arthropods were indirectly affected by genetic aspen differences as their abundance closely followed the abundance of leaf beetle adults and larvae. They were also positively directly related to emitted herbivore-induced plant volatiles. In addition, after the colonization of one half of the experimental field in spring our results revealed a

spatio-temporal shift to the second field half in summer, presumably due to avoidance of tree leaves with induced responses because of the spring infestation.

The second part of the thesis is based on the knowledge that predatory arthropods often use infochemical cues for host finding and host location and that multitrophic interactions are often shaped by such infochemicals. The aim of the two studies of the second part was to investigate the olfaction ability of generalist and specialist predatory insects of volatiles emitted by their prey and the prey's host plants and the effects of these volatiles to the predators. This was tested with a field study and laboratory experiments (olfactometer tests and elctroantennography (EAG)). In contrast to recent studies our results showed that generalist predatory beetles such as *Harmonia axyridis* were attracted by salicylaldehyde, the defense compound of salicin-using leaf beetle larvae. The results further suggest that *H. axyridis* imagines can learn to use salicylaldehyde as an attractant.

The results of EAG experiments indicate that specialist predatory eumenid wasps are able to perceive salicylaldehyde and herbivore-induced plant volatiles and that the olfaction ability reflects the degree of prey specialisation of the different wasp species.

We conclude that direct and indirect effects shape aspen-insect interactions. Aspen influence herbivorous insects directly via plant morphological, chemical and genetic traits as well as indirectly via their spatial distribution and temporal changes. Predators are likewise directly influenced by aspen via emitted herbivore-induced plant volatiles and indirectly via herbivore abundances on the trees. Furthermore, infochemicals emitted by their prey affect generalist and specialist predators in the tritrophic system consisting of *Populus tremula*, specialist leaf beetles, their larvae and their predators.

ZUSAMMENFASSUNG

Für die kommenden Jahre wird ein dramatischer Anstieg des weltweiten Energieverbrauches erwartet. Aufgrund des wachsenden Bedarfs an erneuerbarer Energie richtet sich das Interesse der aktuellen Forschung auf Kurzumtriebsplantagen mit schnell wachsenden und ertragreichen *Salix* (Weiden) und *Populus* (Pappeln) Arten als mögliche Energiequelle. In diesem Zusammenhang erlangt die Europäische Zitterpappel (*Populus tremula*) an Bedeutung, da sie im Stande ist höhere Erträge auf nährstoffarmen Böden und unter ungünstigen klimatischen Bedingungen zu erbringen. Die Europäische Zitterpappel ist eine Pionier-Art und eine der am weitesten verbreiteten Baumart in borealen und gemäßigten Wäldern. In natürlichen Vorkommen bilden Zitterpappeln Habitate für zahlreiche Insekten. Darunter befinden sich auch eine Vielzahl an gefährdeten Arten. Die meisten Studien über Insekten-Pflanzen Interaktionen an Pappel und Weiden konzentrieren sich auf nur zwei trophische Ebenen. Diese Studien untersuchen vor allem den Einfluss von Unterschieden der chemischen Blattinhaltsstoffe auf pflanzenfressende Insekten.

Die vorliegende Arbeit wurde in zwei Teile untergliedert. Ziel des ersten Teils der Arbeit war es Unterschiede in der Morphologie und der emittierten Blattduftstoffe zwischen nah verwanden Zitterpappel Kreuzungen zu charakterisieren. Ein weiteres Ziel war es herauszufinden, inwiefern die morphologischen und chemischen Merkmale der Bäume pflanzenfressende Insekten und deren natürliche Gegenspieler in „natürlichen multitrophischen Interaktionen“ beeinflussen. Für die Untersuchungen wurden zwei verschiedene Freilandversuche etabliert, in denen eng verwandte Zitterpappel Kreuzungen (mit einer genetischen Distanz zwischen 6 – 51 %) gepflanzt wurden Zitterpappeln.

Wie erwartet unterschieden sich die Zitterpappeln der verschiedenen Kreuzungen in ihren morphologischen Merkmalen wie z.B. Baum-Höhe, Blattanzahl, etc. und in chemischen Merkmalen wie phenolischen Glykosiden. Im ersten der beiden Freilandversuche wurde ein negativer Zusammenhang zwischen phenolischen Glykosiden und pflanzenfressenden Insekten (z.B.: *Phratora vulgatissima*) festgestellt. Im zweiten Freilandversuch wurde herausgefunden, dass spezialisierte Blattkäfer und ihre Larven durch unterschiedliche morphologische Baummerkmale beeinflusst werden. Die Abundnaz der Imagines war positiv mit der Anzahl der Blätter pro Baum (Ressource Abundanz) korreliert, wohingegen

ein negativer Zusammenhang zwischen der Baum-Höhe und Blattkäfer Larven gefunden wurde, Dieses Ergebnis steht im Gegensatz zur Pflanzen-Größe Hypothese nach Lawton. Durch Herbivoren induzierte Pflanzen Volatilen unterschieden sich in der Abgabemenge zwischen Frühling und Sommer. Es konnten aber weder Unterschiede zwischen den verschiedenen Kreuzungen noch durch verschiedene Herbivoren Abundanz festgestellt werden. Generalistische räuberische Arthropoden wurden indirekt durch genetische Unterschiede der Zitterpappeln (Kreuzungen) beeinflusst, indem ihre Anzahl positiv mit der Zahl der Herbivoren korreliert ist. Auch wurde festgestellt, dass es einen positiven Zusammenhang zwischen abgegebenen Herbivoren induzierten Pflanzen Volatilen und räuberischen Arthropoden gibt. Zusätzlich zeigten unsere Ergebnisse, dass es auf der Fläche eine räumlich-zeitliche Veränderung des Befalls der Bäume gab. Die im Frühjahr befallenen Bäume wurden von der zweiten Generation von Blattkäfern im Sommer gemieden, da diese vermutlich durch den Befall der ersten Blattkäfer Generation induzierte Resistenzen in den Blättern aufwiesen.

Der zweite Teil der vorliegenden Arbeit basiert auf dem Wissen, dass räuberische Arthropoden oft sogenannte „Infochemikalien“ als Signale nutzen um ihre Beute zu lokalisieren und zu finden. Diese Infochemikalien formen häufig multitrophische Interaktionen. Das Ziel der zwei Studien des zweiten Teils der vorliegenden Arbeit war die Untersuchung der olfaktorischen Reaktion von generalistischen und spezialisierten Prädatoren auf Volatilen die von ihrer Beute abgegeben werden und von Volatilen die von den Wirtbäumen der Beute abgegeben werden. Die Studien wurden in einem Freilandversuch und in Laborexperimenten (Olfaktometer Test und Elektroantennographie (EAG)) durchgeführt.

Im Gegensatz zu anderen Studien zeigten die Ergebnisse, dass generalistische räubereische Käfer wie z.B. *Harmonia axyridis* von Salicylaldehyd, der Abwehr Substanz Salicin sequestrierender Käferlarven, angezogen wurden. Dieses Ergebnis lässt vermuten, dass *H. axyridis* Imagines in der Lage sind zu lernen Salicylaldehyd als Lockstoff zu nutzen.

Die Ergebnisse der EAG Experimente indizieren, dass spezialisierte räuberische Lehmwespen (Eumeninae) in der Lage sind Salicylaldehyd und von Herbivoren induzierte Pflanzen Volatilen wahrzunehmen. Des Weiteren spiegelte die olfaktorische Wahrnehmungsfähigkeit den Grad der Beute-Spezialisierung der drei verschiedenen Wespen Arten wieder.

Zusammenfassend konnte gezeigt werden, dass direkte und indirekte Effekte die Interaktionen zwischen Zitterpappeln und Insekten beeinflussen und formen. Zitterpappeln beeinflussen pflanzenfressende Insekten sowohl direkt durch morphologische, chemische und genetische Merkmale als auch indirekt durch räumliche Verbreitung und zeitliche Veränderungen (induzierte Resistenz). Ähnlich sind Prädatoren von Zitterpappeln direkt durch Herbivoren induzierte Pflanzen Volatilen und indirekt durch die Anzahl von Herbivoren auf den Bäumen beeinflusst. Darüber hinaus beeinflussen Infochemikalien, abgegeben von Beutetieren, generalistische und spezialisierte Prädatoren in dem tritrophischen System bestehend aus *Populus tremula*, spezialisierten Blattkäfern, deren Larven und ihren Prädatoren.

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